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# SABOURAUDIA

## VOLUME 1

Raimond Sabouraud

# RAIMOND SABOURAUD

(1864-1938)

The outstanding names in the history of any branch of science are not always those of the men who made the primary observations. The prevailing climate of opinion and the available techniques greatly affect the contemporary significance attributed to any novel discovery and the historical landmarks are frequently the reputations of subsequent workers who were men of their time, who showed singleness of purpose, and who crystallized ideas which were nearing supersaturation. Raimond Sabouraud, the French dermatologist, was such a man. In the early eighteen-nineties by re-making observations which had been on record for fifty years but not universally accepted he was able to silence finally the view that the association of fungi with ringworm was incidental. Using the recently developed pure-culture techniques he was able to establish convincingly the 'plurality' of the ringworm fungi, and his medical training enabled him to integrate the mycological and the clinical aspects of ringworm. He was stimulated by the advances made by able contemporaries in both medicine and veterinary science and finally in 1910 he codified both his own and their results in the monumental *Les Teignes,* one of the most comprehensive treatments ever given to a group of pathogenic fungi and a monograph to which students of the dermatophytes must still refer.

The dermatomycoses have always been a basic theme of medical and veterinary mycology and at the turn of the century they overshadowed the systemic mycoses by which the dermatomycoses are themselves overshadowed to-day. They served to focus attention on fungi as pathogens of man and higher animals and provided a seed from which an interesting branch of medicine and veterinary science has largely developed. The title of this journal commemorates our debt to Sabouraud.

G.C.A.

# ROBERT WERNER

## 1895-1959

The name Robert Werner doubtless means nothing to the readers of *Sabouraudia*. He was not a mycologist, a botanist or a physician yet without his assistance this review, dedicated to the study of human and animal mycology, would not have appeared to-day. Robert Werner was a highly intelligent and successful business man possessed with an overwhelming sense of generosity. It would not be easy to record the many persons to whose goodwill he contributed by his development of the 'Bunge' group of companies which he directed in 26 countries scattered throughout the 5 continents. It would be easier, but very lengthy to prepare a list of the scientific institutions and teaching organisations which he aided, from the 'Universite Libre' in Brussels, where he was responsible for the founding of the 'Institut de Philologie orientale et slave' to the school for higher education at Gand. It is not possible however to give the names of all the institutions or individuals that he helped, for in most cases, he enforced with the greatest charm, complete secrecy from those he assisted.

When, with our late President, Professor Redaelli, it was agreed that there would be no better way of giving life to ISHAM than by starting a journal, I began to seek financial assistance. I did not have to ask for it. One day while talking with Robert Werner, of whose role as a benefactor I was unaware, he offered to provide immediate help. Before doing so, however, he asked with an embarrassed smile what mycology was and what were the objects of our society. It was then abundantly clear to me that, although he gave willingly and usually anonymously, he did not give without first making certain that the money would be used to good advantage and would contribute, however indirectly, to the welfare and happiness of mankind. Faithful to his promise, Robert Werner gave first 25,000 Belgian francs to assist the society in its debut and on the eve of his death donated a further 300,000 francs to enable us to launch the review which appears to-day.

The committee, and I am sure all the members of ISHAM, are most grateful to Robert Werner for his generosity. He was a great man and a true benefactor to the Arts and Sciences.

R. VANBREUSEGHEM.

# THE INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

At this stage in our Society's history it seems appropriate to review the development of the scientific relationship which exists between medical and veterinary mycologists. These relationships developed naturally through the years and only recently did official organisation seem advisable. I have derived great pleasure in summarizing the events which culminated in the formation of the International Society for Human and Animal Mycology because they show that a tradition, which we shall surely uphold, has already been clearly established.

The discoveries of Bassi in 1835 on the muscardine disease of silkworms and those made shortly afterwards by Remak, Schoenlein and Gruby on the fungus nature of favus and ringworm are a source of immense satisfaction to all medical mycologists. Equally important are the investigations of Langenbeck, Berg, Gruby, and Robin on candidiasis; Virchow on aspergillosis; Rivolta on the epizootic lymphangitis of the equidae, and Carter on the mycetomas. By 1915 most of the mycoses which are known to-day had been discovered.

Sabouraud, to whom we have dedicated our journal, was among those who studied the very common cutaneous mycoses in detail. He rightly occupies a place of honour in the history of medical mycology and tribute was paid to him by Adamson who wrote in the *British Journal of Dermatology* (1895) 'The brilliant researches of M. Sabouraud of Paris, have of recent years attracted attention to the study of the mycology of ringworm and have stimulated research in almost every country where that obstinate affection is epidemic'. Sabouraud's works are noteworthy for their excellent clinical descriptions and mycological observations but his careful and fair evaluation of the contemporary literature is also of considerable value. He gives due credit to earlier workers and in his incomparable *Les Teignes* (1910) he quotes the statement by Gruby (1841) 'Pour reconnâitre la vraie teigne on n'a qu'à la soumettre au microscope'. In 1910 Brumpt devoted some 200 pages of his comprehensive *Précis de Parasitologie* to medical mycology. If there is any criticism of this period it is that since the deep mycoses were infrequently encountered human mycology was too closely associated with dermatology.

From 1915 until about 1930 was a period of estimates. Specialized laboratories were almost non-existent and in most cases insufficient data were given in the descriptions of the numerous pathogenic species which were reported. Great emphasis was placed on the macroscopic characters and overmuch importance given to minor differences. It was during this period that Castellani used biological properties as a basis for identification of the pathogenic yeasts. Clinical experience continued but there was little evaluation of results and the literature was scattered and difficult to interpret. The study of the mycoses became more and more difficult. The consequences of the work of this period are reflected by Ramsbottom in the report of the Second International Congress for Microbiology. He said 'On the other hand, the taxonomy of the fungi of medical mycoses is in a bewildering chaos. Many forms have been named in a haphazard manner, with the result that a subject intrinsically difficult has been made repellent to the majority of medical pathologists'. A marked aversion to the study of medical mycology was indeed frequent and persists to a certain extent even to-day. The difficulties which were encountered caused many workers to avoid the study of medical and veterinary mycology and to pass their problems to those who were interested in pathogenic fungi. Because of this isolation, medical and veterinary mycology acquired some autonomy.

During the decade 1930-40 a number of new laboratories, which specialized in the study of the causal organisms of the mycoses, were founded. Valuable work was done by Langeron at the Faculté de Medécine, Paris, and a number of workers such as Ota, Milochevitch, Talice, and Guerra acquired their mycological training there. In the United States medical mycology had already advanced greatly as is shown by the publication in 1932 of Jacobson's *Fungus Diseases* and in 1935 of C. W. Dodge's *Medical Mycology*. The latter invaluable work which reflects the situation at this time constitutes an excellent bibliographical guide and a source of reliable descriptions of pathogenic fungi. The names Benham, Henrici, Hopkins, Conant, DeLamater, Emmons, Lewis, Moore, Schwartz, and others so familiar to all of us appear in the literature of this period. Benham's paper 'Certain Monilias Parasitic on Man' in the *Journal of Infectious Diseases* (1931) is considered a classic and her use of morphological, biological and serological methods to create order in this difficult group of fungi has proved invaluable. In Latin-America also much more attention was now being paid to fungus diseases. In Brazil by da Fonseca and de Arêa Leão at the Instituto Oswaldo Cruz, Rio de Janeiro, and by de Almeida in São Paulo; in the Argentine by Negroni and Niño; in Puerto Rico by Carrión; In Uruguay by Talice and in Venezuela by O'Daly, Guerra and Montemayor. The deep mycoses were frequently diagnosed in the New World. In Europe during this decade the Italian school of Redaelli and Ciferri became prominent and the Centro de Micologia Umana e Comparata was founded in Pavia.

Frequent communication and co-operation among the various laboratories was now commonplace and invaluable service was being given by centres for type-culture collections, which were also contributing to taxonomy. The work of the Centraalbureau voor Schimmelcultures in Baarn is worthy of particular mention and medical mycologists are deeply indebted to Dr. Johanna Westerdijk the director at that time. The monographs, *Die Anaskosporogenen Hefen* by Lodder (1934) and by Diddens and Lodder (1942) constitute fundamental contributions to the taxonomy of pathogenic and nonpathogenic yeasts. Publications were now becoming more numerous and criticism was proving effective.

In 1938 Redaelli and Ciferri founded the journal *Mycopathologia* on the cover of which figured a long list of collaborators from many countries. Thus the first tangible bond among medical mycologists was created. Redaelli and Ciferri, by their further activities assisted the formation of our society.

In other ways, co-operation among medical mycologists was evident. In 1939, a considerable number of papers on human and animal mycology were read before the Third International Congress for Microbiology in New York, and there was a meeting of all those interested to discuss the problem of the nomenclature of the so-called 'monilias'. This informal gathering, held in Benham's laboratory at Columbia University, was probably the first international meeting of specialists in medical mycology.

The considerable advances in the knowledge of the mycoses made by this time are illustrated by Langeron's contribution to *Nouvelle Pratique Dermatologique* (1936) and by such works as *Micologia Medica* by F. de Almeida (1939) and *Introduction to Medical Mycology* by Lewis and Hopper (1939).

Progress, particularly in the knowledge of the deep mycoses was more rapid during 1941-1950. Worthy of special mention are the books *Granulmatosi Fungine dell-Uomo nelle Regioni Tropicale e Subtropicale* by Redaelli and Ciferri (1942), Henrici's *Molds, Yeasts and Actinomycetes* revised by Skinner, Emmons and Tsuchiya (1947) and the *Manual of Clinical Mycology* by Conant, Martin, Baker, Smith and Callaway (1944). The latter, essentially a practical book, had a remarkable influence and illustrates the importance attached to human mycology in the United States of America. A second edition appeared in 1954. The discovery of clinical and sub-clinical non-evolutive forms of coccidioidomycosis by Dickson and Gifford and similar discoveries in histoplasmosis by Palmer, by Christie and Petersen, and by Furcolow, greatly increased the number of diagnosed cases. The finding in the soil of the pathogenic fungi *Coccidioides immitis*, *Histoplasma capsulatum* and *Allescheria boydii* opened new fields to epidemiological studies and stimulated a search for mycotic infections in wild animals. In 1946 a new form of histoplasmosis was described by J. T. Duncan and the completed work was reported in a posthumous paper entitled 'Tropical African Histoplasmosis' in the *Transactions of the Royal Society of Tropical Medicine and Hygiene* (1956). Certain Belgian workers have also contributed greatly to the investigation of this disease.

In 1943 the Commonwealth Mycological Institute began the publication of abstracts in the *Annotated Bibliography of Medical Mycology*, now known as the *Review of Medical and Veterinary Mycology*. It has become an indispensable reference work and I acknowledge with gratitude the part played by our late fellow-member Dr. James T. Duncan in the establishment of this publication.

In the medical mycology section of the Fifth International Congress for Microbiology in Rio de Janeiro in 1950 many papers were presented, and formal meetings were arranged by Dr. W. J. Nickerson on behalf of the Committee on Medical and Veterinary Mycopathology of the International Association of Microbiologists. Many problems of nomenclature were discussed and the conclusions were published in the *International Bulletin of Bacteriological Nomenclature and Taxonomy* (1952). In Britain the Medical Mycology

Committee of the Medical Research Council published in 1949 the *Nomenclature of Fungi Pathogenic to Man and Animals* and this was revised in 1958.

During the last decade the amount of research and the co-operation between workers has increased. The widespread use of antibiotics, corticosteroids and other drugs has been reputed to cause unusual, severe or benign cases of candidiasis as well as an increase in the frequency of deep evolutive mycoses. The discovery of drugs and antibiotics possessing a fungistatic action and which are useful in the treatment of the deep mycoses has made exact diagnosis of these a practical and often vital issue. Early diagnosis has now become a necessity and we must resort frequently to immunological methods, which are also indispensable for the prognosis and evaluation of specific therapy. Quite recently the treatment of ringworm with an orally administered antibiotic has proved effective. As time passes the need for more research into the pathogenesis and immunology of the mycoses becomes more and more evident. There is a need also for careful study of the physiology of the pathogenic fungi. The search for pathogenic fungi in the soil as carried out by Ajello and his colleagues and by Furcolow has contributed to our knowledge of the cause of epidemics of histoplasmosis. There is a need for further investigations on the ecology of the causal organisms of deep mycoses. New species of dermatophytes have been isolated from the soil by Vanbreuseghem and by Ajello and have later been found in association with animals.

Veterinary mycology is now a rapidly growing subject. Specialized laboratories have been formed. The scientific and economic importance of mycotoxicosis due to mouldy food-stuffs, mycotic bovine abortion, mycotic mastitis, mycotic dermatitis in sheep, and the like are now fully appreciated. Advances have been made by Van Uden and others in the knowledge of the yeasts of the intestinal tract of warm-blooded animals. A new form of histoplasmosis has been described in African monkeys by Mariat and Segretain and the latter has also described a systemic penicilliosis in a wild rodent. A book *Fungal Diseases of Animals* with extensive bibliographical references has been produced by Ainsworth and Austwick.

As pointed out by Ciferri in his *Manuale di Micologia Medica* (1958) the gap between medical and classical mycology is not now as wide as when Vuillemin (1911) proposed his useful classification.

In 1952, Ainsworth began the preface of his little introductory book *Medical Mycology* with the following words, 'Both medical men and mycologists incline to the view that medical mycology is a confused and somewhat esoteric subject. Medical mycologists are not of this opinion. They would maintain that the apparent confusion must in large measure be attributed to the neglect into which this branch of medicine and mycology has fallen and the failure to appreciate the results of modern research'. Some of the discoveries of the last decade have resulted in greater attention being paid to medical and veterinary mycology. Regional groups of specialists or national societies of medical and veterinary mycology have been organized in England (1953), in France (1956) and in Japan, etc. A journal *Mykosen* is edited in Germany. A number of symposia and conferences have been held by the New York Academy of

Science and by other institutions in the United States. In London, in 1957 the Wellcome Museum of Medical Science organized a large new section on medical mycology. The number of medical mycology courses held in American and in European institutions has increased and in this connection I mention particularly the annual course given at the Institut Pasteur, Paris. A special committee for the purpose of encouraging and co-ordinating investigations has been formed in Venezuela.

These facts demonstrate that great changes have taken place in our field during the last few years. The relationships between the specialists in medical and veterinary mycology has also been significantly improved. During the meetings at the Fifth International Congress for Microbiology (1950) some members expressed the wish for an official organization. It was no surprise, therefore, when at a friendly gathering during the next congress in Rome in 1953, Ciferri and Redaelli proposed that a Society for Human and Animal Mycology should be formed. The Society was founded a year later, on the 6 July, 1954, at the Eighth International Botanical Congress in Paris and Professor Redaelli was elected President. It is worthy of mention that this was the first time a section on medical mycology had been included in an International Botanical Congress and that this innovation was repeated at the next congress in Montreal in 1959.

Despite the blow which Redaelli's death, in October 1955, dealt to the Society it continued to flourish. An annual bulletin was published and a formal meeting was organized in Lisbon during the Sixth International Congresses of Tropical Medicine and Malaria. We are greatly indebted to Professor Vanbreuseghem, the general secretary, for carrying us through these early years.

The great loss of Redaelli is keenly felt by all of us. He was cultured and artistic and a able scientist who, by virtue of his friendly and attractive personality, did a great deal to strengthen the ties and further co-operation among medical mycologists. I believe we can best show our gratitude to Piero Redaelli and honour his memory by furthering the work of the society which he did so much to create. By the efforts of the present executive and editorial committees we are now furnished with a constitution and a journal. It is my sincere wish that the society will go forward in strength and purpose in the years ahead.

JUAN E. MACKINNON.
*President.*

# YEASTS FROM HUMAN SOURCES

D. W. R. Mackenzie,

*Experimental Mycoses Unit**,
*Department of Botany, Edinburgh University.*

Specimens of urine, sputum, throat swabs, stools, high vaginal swabs, wound swabs or discharges, and other sources were examined for the presence of yeasts : 246 isolates representing 23 species were obtained from 1004 specimens examined. Qualitative and quantitative aspects of the yeast flora of each source are considered. *Candida albicans* was the most common species and was isolated on 141 occasions (56 % of the total number of yeasts). *Torulopsis glabrata* was shown to be a significant member of the flora of the sources examined, and was the most common species isolated from urine. Other common yeasts were *Rhodotorula mucilaginosa* and *C. tropicalis.*

Between June, 1957 and Aug., 1958 a survey of the yeasts associated with various human sources was made at the Experimental Mycoses Unit, Department of Botany, University of Edinburgh, prior to the establishment of a mycological diagnostic service for hospitals in the Edinburgh district. It was designed to determine the incidence of specific yeasts, to build up a culture collection of representative species, and to design routine methods for isolation and identification.

The samples for mycological examination were obtained at random from a cross-section of hospital patients. Many of the samples were taken for routine bacteriological examination and a proportion of these samples was shown at the hospital laboratory to be free from pathogenic bacteria. No attempt was made to correlate the presence of an organism with any clinical symptom or disorder, nor were the species studied in relation to past or present administration of therapeutic agents.

## Materials and Methods

Specimens were obtained from patients in 14 Edinburgh hospitals. All samples were treated in the same manner. Small amounts of the material were streaked over the surface of an agar plate, after preliminary preparation of a smear for microscopic examination. Isolation of yeast colonies was carried out on 2·5 % malt-agar containing penicillin (20 units/ml.) and streptomycin (40 units/ml.). Inoculated plates were incubated at room temperature for 7 days and at 37° C. for a further 7 days. Developing colonies were transferred to malt-agar slopes and stock cultures obtained following streak purification on a malt-agar plate. The ability of each isolate to tolerate or grow at 37° C. and the appearance and number of colonies developing on the isolation plate were recorded. Identifications were made by reference to Lodder & Kreger-van Rij (1952). Full details of the methods employed are described by Mackenzie (1958).

## Results

The results are summarised in table I.

---

* Present address : Department of Microbiology, Queen's University of Belfast.

| | Sources | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Urine* | *Sputum* | *Stool* | *Throat* | *Vagina* | *Wounds etc.* | *Others* | *TOTAL* |
| No. of samples examined .. .. .. | 182 | 174 | 103 | 79 | 150 | 203 | 113 | 1004 |
| No. of samples yielding yeasts .. .. | 17 | 120 | 35 | 43 | 13 | 17 | 2 | 247 |
| % age .. .. .. .. .. .. | 9·3 | 68·9 | 33·9 | 54·3 | 8·7 | 8·4 | 2·7 | 24·7 |
| No. of species of yeasts .. .. .. | 8 | 18 | 6 | 9 | 5 | 9 | 1 | 23 |
| 1. SACCHAROMYCETACEAE | | | | | | | | |
| *Hansenula anomala* Hansen et P. Sydow .. | — | 2 | — | — | — | — | — | 2 |
| *Pichia membranaefaciens* Hansen .. .. | — | 2 | — | — | — | — | — | 2 |
| *Saccharomyces carlsbergensis* Hansen .. .. | — | 1 | — | — | — | — | — | 1 |
| *Saccharomyces cerevisiae* Hansen .. .. | — | 4 | — | — | — | — | — | 4 |
| *Saccharomyces cerevisiae* var. *ellipsoideus* (Hansen) Dekker .. .. .. .. .. | — | 1 | — | — | — | — | — | 1 |
| *Saccharomyces fragilis* Jörg. .. .. .. | 1 | — | 1 | 1 | — | — | — | 3 |
| 2. CRYPTOCOCCACEAE | | | | | | | | |
| *Candida albicans* (Robin) Berkhout .. .. | 3 | 70 | 28 | 27 | 7 | 6 | — | 141 |
| *Candida guilliermondii* (Cast.) Langeron et Guerra .. .. .. .. .. | — | — | — | — | 1 | — | — | 1 |
| *Candida krusei* (Cast.) Berkhout .. .. | — | 2 | — | 1 | — | — | — | 3 |
| *Candida parapsilosis* (Ashf.) Langeron et Talice | 1 | 2 | 1 | 2 | — | 3 | — | 9 |
| *Candida robusta* Diddens et Lodder .. .. | — | 1 | — | — | — | — | — | 1 |
| *Candida tropicalis* (Cast.) Berkhout .. .. | — | 7 | 2 | 2 | — | 1 | — | 12 |
| *Candida zeylanoides* (Cast.) Langeron et Guerra | 1 | 1 | — | 3 | 1 | 1 | — | 7 |
| *Cryptococcus albidus* (Saito) Skinner .. .. | 1 | 1 | — | — | — | 2 | — | 4 |
| *Cryptococcus diffluens* (Zach.) Lodder et Kreger Van Rij .. .. .. .. .. | — | — | — | — | — | 1 | — | 1 |
| *Cryptococcus laurentii* (Kufferath) Skinner .. | — | 1 | — | — | — | — | — | 1 |
| *Cryptococcus neoformans* (Sanf.) Vuill. var. *uniguttulatus* (Zach.) Lodder .. .. | — | 1 | — | — | — | — | — | 1 |
| *Rhodotorula mucilaginosa* (Jörg.) Harrison .. | 4 | 7 | 1 | 2 | 1 | 1 | — | 16 |
| *Rhodotorula rubra* (Demme) Lodder .. .. | — | — | — | — | — | 1 | — | 1 |
| *Torulopsis candida* (Saito) Lodder .. .. | — | 1 | — | — | — | — | — | 1 |
| *Torulopsis famata* (Harrison) Lodder et Kreger Van Rij .. .. .. .. | 1 | — | — | — | — | — | — | 1 |
| *Torulopsis glabrata* (Anderson) Lodder et De Vries .. .. .. .. .. | 5 | 15 | 2 | 4 | 3 | — | 2 | 31 |
| *Trichosporon capitatum* Diddens et Lodder .. | — | — | — | — | — | 1 | — | 1 |
| *Trichosporon cutaneum* (de Beurm., Gougerot et Vaucher) Ota .. .. .. .. | — | — | — | 1 | — | — | — | 1 |
| *Taphrina* sp. .. .. .. .. .. | — | 1 | — | — | — | — | — | 1 |

TABLE I.—YEASTS ISOLATED FROM MAN.

*Urine.* It is of interest to note that the yeast most commonly isolated from urine was *Torulopsis glabrata.* Although reports of this yeast are scarce in the literature, Artagaveytia-Allende & Garcia-Zorron (1957) have reported its presence in the human vaginal tract. Orie (1952) has isolated a strain from urine. It is perhaps significant that all 5 isolations of *T. glabrata* were from female patients, and it may be that in most of the cases its presence in the urine was due to contamination from tissues outside the urinary tract. In one instance, however, this species was recovered from a catheter specimen, and this would indicate that the yeast originated from the urine rather than from the vagina.

Of the remaining 11 yeast isolates, 8 were from female patients, and in none of these was a catheter employed, hence the possibility exists that they were members of the mycoflora of the external genitalia or vagina. Two of the 3 isolates of *Candida albicans* were from male patients. This yeast has been reported as causing an infection of the urinary tract (Engelhard, 1948; Taylor & Rundle, 1952). *Rhodotorula mucilaginosa* is common in human and other sources : like *T. glabrata* it can possibly be regarded as a normal member of the genital flora in the human. Three of the 4 cultures obtained in the present survey were from female patients.

All isolates with the exception of *Cryptococcus albidus* were capable of growth at 37° C.

*Sputum.* Several studies on the mycoflora of sputum have been made (Ying, 1936; Greer & Gemoets, 1943; Kurung, 1947; Honorato, 1948; Dietrichson, 1950; Brygoo, 1951; Dietrichson, 1954; Armstrong & Hall, 1956; Helms, 1956a, b.). The species recorded and their relative frequencies differ, but a general pattern of distribution is common to them all. *C. albicans* is invariably the most common, followed by a group comprising *C. tropicalis, C. krusei,* and *C. parapsilosis*. Other species occasionally found include *C. pseudotropicalis, C. lipolytica, C. macedoniensis, C. brumptii* and *C. guilliermondii.*

In the present survey, *C. albicans* was the most common species, representing 57·6 % of the total number of yeasts isolated from sputum. A feature of unusual interest is the exceptionally high incidence of *T. glabrata* which was isolated on 15 occasions (12·2 %). This organism has not been considered until now as a significant member of the yeast flora of mouth, oropharynx or respiratory tract, and the number of occasions on which it has been recorded is small (Black & Fisher, 1937 : Dietrichson, 1954; Artagaveytia-Allende & Garcia-Zorron, 1957). It should be noted that the majority of samples received for examination were from patients suffering from a disorder of the respiratory system. Many, but not all, were suffering from bronchitis or suspected pneumonia whilst the remainder had diagnoses such as heart failure, pulmonary embolism, emphysema, etc., where the presence of pathogenic organisms was not suspected.

The number of organisms which have been recorded from sputum is large, and it is generally recognised that many of these are incidental, having been acquired accidentally from the environment. It is well known that yeasts are common on a variety of foodstuffs, and a number have been recorded from the atmosphere (Connell & Skinner, 1953; Di Menna, 1955). Yeasts occurring in the present survey whose sources are probably exogenous include the ascosporogenous *Saccharomyces, Hansenula* and *Pichia,* all of which have been isolated from vegetable sources. *Cryptococcus albidus* and *C. laurentii* are both normally found in saprophytic environments, although the former has been recorded from sputum in Holland (Orie, 1946). *Torulopsis candida* and *Candida robusta* are probably not significant members of the flora of sputum, and these 2 isolates, together with *Cryptococcus albidus* and *C. laurentii* were incapable of growth at 37° C.

*Throat swabs.* The distinction between throat and sputum samples may be considered arbitrary, but certain differences are apparent when the 2 sources are compared. This difference is quantitative rather than qualitative, since all the species occurring in the throat were also represented in the sputum. Of the

17 species occurring in the sputum, only 6 were isolated from throat samples. Their frequency was also much lower, 34 % as against 68·4 %. It is likely that the throat harbours fewer yeasts than the mouth. Many of the yeasts in the mouth have presumably been acquired from an external source : within the mouth they may remain viable for varying lengths of time on particles of food or the original substrate in crevices between the teeth or beneath dentures. The organisms acquired accidentally may be capable of resisting for a time the changes in the environment, including a higher temperature, the presence of salivary enzymes, and the competition with normal commensals. Ultimately, their inability to survive these conditions would be reflected in a reduction of their numbers. Those organisms which are capable of surviving would be better adapted to their new environment and could possibly become established as true members of the flora of the mouth or throat. The results suggest that the throat is a less favourable environment for their growth and propagation than the mouth. All the yeasts isolated from the throat were capable of growth at 37° C.

*Stool.* A number of studies on the yeasts of the human intestinal tract have been made (Colonnello, 1943; Solari & De Fernandez, 1948; Reyes. 1955; Windisch & Staib, 1955; Ochoa & De Los Angeles, 1956; Mehnhert, 1957; Artagaveytia-Allende & Garcia-Zorron, 1957). Many deal specifically with the incidence of *C. albicans.* Negroni (1955) has recorded the presence of yeasts in 36 out of 50 specimens of faeces. He reports the species most commonly associated with this site to be *C. krusei* followed by *C. parakrusei* (=*C. parapsilosis*), *C. albicans, C. zeylanoides, C. tropicalis,* and *C. chalmersi* (=*C. parapsilosis*). Both Windisch & Staib (1955) and Mehnhert (1957) have reported a different pattern. The former report the presence of yeasts in 37 % of 1,158 samples of human faeces, whilst Mehnhert has recorded an incidence of 26 %. The figure obtained during the present survey was 54·3 %. Windisch & Staib and Mehnhert reported that *C. albicans* was the most common species, and this was also noted in the present survey. *C. krusei* is frequently present in the human intestinal tract, but was found on only 1 occasion in the present study. *C. parapsilosis, Trichosporon cutaneum* and *C. zeylanoides* (Windisch & Staib, 1955; Negroni, 1955; Mehnhert, 1957) have all been reported from faeces and each of these species was isolated from this source during the present study. *Saccharomyces fragilis* has also been reported from this site (Lodder & Kreger-van Rij, 1952), and, although members of the ascosporogenous yeasts have frequently been isolated from faeces, they appear to be incidental rather than normal inhabitants of the gut. *Rhodotorula mucilaginosa* has also been reported from faeces and 2 of the strains studied by Lodder & Kreger-van Rij (1952) were isolated from this source.

*Torulopsis glabrata* was again prominent amongst the species isolated in the present survey. This organism was originally isolated from human faeces (Anderson, 1917) but it has seldom been reported since from this source. Parle (1957) isolated 3 strains in the course of his investigation on the yeasts occurring in the alimentary tract of several mammals. All the organisms isolated from stool samples were capable of growth at 37° C.

*Vagina.* Studies on the human vaginal flora (Vaccaro & Ferrada Urzua, 1952; Jones, Wood, Bishop & Donoho, 1955; Bittencourt & Camara, 1956; Halde &

Aragon, 1956; Artagaveytia-Allende & Garcia-Zorron, 1957) have consistently revealed that the most common yeast in the vagina is *C. albicans*, which is often associated with vaginitis. The vaginal specimens received for mycological examination were almost exclusively high vaginal swabs which represented routine examination of the upper portion of the vagina following parturition. Such swabs were all obtained during the puerperium and are thus not comparable to those made from the vagina at other times. Following parturition, the environment of the vagina is considerably altered. This is reflected in the reduced number of isolates made from this type of vaginal swab when compared with the results obtained by other workers.

*Wounds, etc.* Seventeen isolates were obtained from 203 cultures examined. Samples were taken from surgical and other wounds, including surface swabs and pus from discharging sinuses, lesions, boils, abscesses, carbuncles, stitch abscesses, and bed sores. Most of the samples were obtained from septic wounds or sores occurring on various sites such as the heel, ear, breast, finger, leg, scrotum, eye, hand, neck, scalp and groin. Less than 5 % of the total were from internal abscesses or ulcers, and no yeasts were isolated from these samples.

The superficial position of the majority of the wounds or sores together with the presence of dead tissue suggested that from this material a number of exclusively saprophytic forms may have been isolated. It is interesting to note that of the species isolated from these sources only *C. albicans, C. parapsilosis* and *C. tropicalis* were capable of growth at 37° C. None of the species of *Cryptococcus* was able to grow on cultures maintained at this temperature.

Yeasts have occasionally been reported from pus (Catanei, 1950; Lamy, Aussannaire, Jammet & Caramanian, 1953) and pathological conditions of the human skin (Serrano, 1940). Both *C. albicans* and *C. parapsilosis* have been reported from cutaneous lesions (Ochoa & Sandoval, 1943). Drouhet (1957) considers that the isolation of *C. albicans* from cutaneous lesions is of pathological significance, since it is the only species of *Candida* not found in the saprophytic state on the skin, but only on the mucous membranes. If this is so, it is possible that the isolates of this organism in the present survey may have been implicated in the sepsis. In this respect it probably differed from the other species isolated from wounds, etc., where there was no evidence to suggest that those yeasts might have been actively associated with inflammation. From the methods used and the results obtained, it was clear that the presence of yeasts in septic foci was exceptional. Over 92 % of the plates examined were negative for yeasts, although bacteria were invariably present.

*Other sources.* The 2 isolates of *T. glabrata* were from a case of 'sticky eyes' in a male infant, 9 days old, and from a rheumatic nodule in a 71 years old female patient. The sources examined in this heterogeneous group consisted primarily of routine swabs from ear, nose and eye, and swabs from infected nail beds (onychia), skin scrapings, bursitis, etc.

## DISCUSSION

The results show that only 4 species can be regarded as relatively common. *Candida albicans* accounted for 56 % of the total number of yeasts isolated, followed by *Torulopsis glabrata* (12·4 %), *Rhodotorula mucilaginosa* (6·1 %) and *Candida tropicalis* (4·8 %).

In the present survey, *C. albicans* was isolated from stools, urine, sputum, throat swabs, and from wound discharges. It was the yeast most frequently isolated from all sources, with the exception of urine, where *T. glabrata* was most common. Since its original isolation *T. glabrata* has been isolated from a number of sources. Reports of its occurrence were originally from human sources only, but later studies revealed its association with animals. It has been isolated by Phaff, Mrak & Williams (1952) from shrimps, and by Parle (1957) from the mammallian alimentary tract. Recca & Mrak (1952) have isolated a strain from concentrated orange juice and a single report of its isolation from garden soil in Holland has been made (Battley, 1956).

The pathogenic status of this yeast is uncertain, and results of animal inoculation tests have been inconsistent. From studies made by Black & Fisher (1937), Lodder & De Vries (1938), Lodder & De Minger (1947), and Lopez-Fernandes (1953) it would appear that the species is a marginal pathogen in laboratory animals, the degree of pathogenicity possibly being determined by the strain of yeast employed and the susceptibility of the individual host.

The unusually, and unexpectedly, high incidence of *T. glabrata* is perhaps the most interesting feature of the present survey, particularly since it was isolated from all but one of the sources examined. This yeast has never been recorded from the atmosphere, and settling plates exposed at intervals in the laboratories where isolation and incubation were carried out failed to reveal its presence. The possibility that aerial contamination accounted for the large number of isolates could therefore be largely, if not entirely, discounted.

Wickerham (1957) suggested that this yeast is apparently increasing in incidence, and believes that this is a sequel to the widespread use of broad-spectrum antibiotics, comparable to the rise in number of infections caused by *C. albicans*. Haley (1959) has provided further evidence of its increase in incidence, a result the present studies would appear to confirm.

## RÉSUMÉ

On a examiné des spécimens d'urine, d'expectorations, de tampons d'ouate des prélèvements dans la gorge, de selles, de tampons vaginaux, de tampons ou d'écoulements de blessures, et d'autres sources, pour y trouver des levures. Sur 1004 specimens, on a obtenu 246 isolés représentant 23 espèces. On considère des aspects qualitatifs et quantitatifs de la flora de levure de chaque source. *Candida albicans* s'est révélé l'espèce la plus commune (141 isolés; 56 p.c. du nombre entier de levures.) *Torulopsis glabrata* s'est trouvé un membre important de la flore des sources examineés, étant l'espèce le plus souvent isolée de l'urine. Autres levures frequentes furent *Rhodotorula mucilaginosa* et *C. tropicalis*.

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# PATHOGENICITY OF *C. ALBICANS* AND *C. TROPICALIS*

H. F. Hasenclever and W. O. Mitchell

*Department of Health, Education and Welfare, Public Health Service, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda 14, Maryland.*

Most strains of *C. tropicalis* possess a considerable degree of virulence for mice. When compared as a group, the strains of *C. tropicalis* were not quite as virulent as were the strains of *C. albicans* studied, and showed very little or no pathogenicity for rabbits.

*In vivo* multiplication rates showed that for both species most of the yeast cell or yeast cell aggregate proliferation occurred in kidney tissue. *C. tropicalis* differed in that there was an early yeast cell growth in the brains of infected mice, 2–4 days after injection, and with a subsequent decrease of viable yeast cells. Histologic sections of brain tissue confirmed the cultural observations.

Early studies of the pathogenicity of members of the genus *Candida* indicated that *C. albicans* was the only pathogenic species. Redaelli (1924) and Benham (1931) found that *i.v.* injection of this yeast into rabbits caused a lethal infection, whereas, a number of other species of this genus were nonpathogenic for rabbits. More recent studies by several different investigators have shown that the mouse is subject to lethal infection by *C. albicans*. Hasenclever (1959) compared the virulence of this yeast for mice and rabbits and found mice to be equally or more susceptible than rabbits. He observed that *C. tropicalis* demonstrated some lethality for mice, but not for rabbits. Mankowski (1957) had earlier noted that *C. albicans*, *C. stellatoidea*, and *C. tropicalis* were pathogenic for mice.

We are reporting mortality rates observed after injection of 52 strains of *C. albicans*, and 14 strains of *C. tropicalis*, in mice and 4 strains of *C. tropicalis* in rabbits. Multiplication rates of *C. albicans* and *C. tropicalis* in the tissues of infected mice are also shown.

## MATERIALS AND METHODS

Female Swiss white mice weighing 15–17 g. were used. Five groups of mice, each containing 5 mice, were utilized for determining the mortality rate for each *C. albicans* strain. Yeast cell suspensions containing $10^7$, $10^6$, $10^5$, $10^4$, and $10^3$ cells/0·2 ml. were prepared, and each dilution was injected *i.v.* into a separate group of mice, 0·2 ml./mouse. The animals were observed for 35 days, and as deaths occurred, they were recorded. For studies with the strains of *C. tropicalis*, 10 mice were used for each group, and suspensions of $10^8$, $10^7$, $10^6$, $10^5$, $10^4$, and $10^3$ cells/0·2 ml. were utilized. Otherwise, the injection procedures were the same as described for *C. albicans*.

Four strains of *C. tropicalis* were injected into rabbits. Six male albino rabbits weighing approximately 2 kg. each were used for each strain. Three suspensions containing fivefold differences in yeast cell numbers were used, and 2 rabbits were injected *i.v.* with each dilution. The suspensions contained $5 \times 10^8$, $10^8$ and $2 \times 10^7$ cells/ml., and 1 ml. was injected into the animal.

The suspensions were prepared from cultures grown 24–48 hr. on 2 % glucose, 1 % neopeptone agar. Most of the strains of both species utilized in this study were recently isolated, although some have been artificially cultured for several years. The identity of all strains was proven by morphological, fermentation, and carbon assimilation tests.

Yeast cell numbers were determined by direct count in a Levy haemocytometer chamber.

The results of mortality studies are expressed as the number of yeast cells causing death in 50 % of the mice ($LD_{50}$), and was calculated using the Reed Muench method (1938).

*In vivo* multiplication rates were determined by sacrificing mice infected with a standard number of yeast cells at designated time intervals. Four mice were sacrified at each interval, and the organs of 3 animals were used to determine the viable yeast cell or mycelial aggregate number, while the organs of the fourth were used to make histologic sections. Blood (heparinized), spleens, kidneys, lungs, and brains were collected, the similar organs were pooled, *i.e.,* 3 spleens, 3 pairs of kidneys, etc., and the different organs were ground with saline separately in sterile mortars and pestles. The tissue-saline ratio after grinding was 1:9, v/w respectively. Serial tenfold dilutions were made of the ground 1:10 tissue suspensions, and pour plates, with 2% glucose trypticase soy agar of the appropriate dilutions were made. The colonies were counted after 1–2 days of incubation at 30°C., and the number of viable yeast cells or mycelial aggregates per 0·1 g. of tissue was calculated. Mice used in these experiments were injected *i.v.* with a number of yeast cells expected, on the basis of prior test, to produce death in all animals. The last mice were killed and the final plating procedure in each experiment was done when the surviving mice were in the terminal stages of disease.

Two strains of *C. albicans,* B311 and 792, were studied. For strain B311, $10^5$ yeast cells were injected into each animal, and for strain 792,$2 \times 10^5$ cells/mouse were used. Three strains of *C. tropicalis,* 20, 48, and 114 were tested, and a dose of $10^6$ yeast cells/mouse was injected.

The histologic sections were stained by the Bauer method as modified by Lillie (1948).

## Results and Discussion

Data obtained from injection of the *C. albicans* strains are shown in table 1. The left column indicates the range of $LD_{50}$, the middle column the number of strains whose $LD_{50}$'s fell within that respective range, and the right column shows the % age of strains within the category. Sixty-one % of the strains had $LD_{50}$ of from $10^4$–$10^5$ yeast cells, and 94 % fell within the hundredfold range of $10^4$–$10^6$ yeast cells.

Table 2 shows the results of $LD_{50}$ calculations from mice after injection with *C. tropicalis.* Twelve of the 14 strains (86 %) fell within the same $LD_{50}$ range as for most of the *C. albicans* strains, *i.e.,* $10^4$–$10^6$ yeast cells. Two strains, however, were less virulent than any of the *C. albicans* strains.

Because of the surprising degree of virulence for mice, displayed by most of the strains of *C. tropicalis,* studies with rabbits were done. Three of the most virulent strains and 1 relatively avirulent strain were tested. The mortality results are shown in table 3. According to Benham (1931) 1 ml. of a 1 % suspen-

| *$LD_{50}$ (No. of yeast cells)* | *No. of strains* | *%* |
|---|---|---|
| $10^6$– $10^7$ | 2 | 4 |
| $10^5$– $10^6$ | 17 | 33 |
| $10^4$– $10^5$ | 32 | 61 |
| $10^3$– $10^4$ | 1 | 2 |
| TOTAL | 52 | 100 |

TABLE 1.—VIRULENCE OF 52 STRAINS OF *C. albicans* FOR MICE.

| *$LD_{50}$ (No. of yeast cells)* | *No. of strains* | *%* |
|---|---|---|
| $10^7$– $10^8$ | 2 | 14 |
| $10^5$– $10^6$ | 6 | 43 |
| $10^4$– $10^5$ | 6 | 43 |
| TOTAL | 14 | 100 |

TABLE 2.—VIRULENCE OF 14 STRAINS OF *C. tropicalis* FOR MICE.

sion of *C. albicans* cells would kill rabbits. Such a suspension contains approximately $10^8$ yeast cells. None of these strains of *C. tropicalis* killed rabbits at that dosage. Hasenclever (1959), moreover, found 1 strain of *C. albicans*, typical in every other respect, that did not demonstrate that degree of lethality. Table 4 shows the mortality rate in mice produced by these same 4 strains of *C. tropicalis*.

| *Strain No.* | *No. of Cells Injected* | | |
|---|---|---|---|
| | $5 \times 10^8$ | $10^8$ | $2 \times 10^7$ |
| 48 | $\frac{1}{2}$ | $\frac{0}{2}$ | $\frac{0}{2}$ |
| 114 | $\frac{0}{2}$ | $\frac{0}{2}$ | $\frac{0}{2}$ |
| 3166 | $\frac{0}{2}$ | $\frac{0}{2}$ | $\frac{0}{2}$ |
| B1211 | $\frac{1}{2}$ | $\frac{0}{2}$ | $\frac{0}{2}$ |

Numerator indicates the number of rabbits dead.
Denominator indicates the number of rabbits injected.

TABLE 3.—MORTALITY RESULTS IN RABBITS WITH 4 STRAINS OF *C. tropicalis.*

| *Strain No.* | *No. of Cells Injected* | | | | |
|---|---|---|---|---|---|
| | $10^8$ | $10^7$ | $10^6$ | $10^5$ | $10^4$ |
| 48 | $\frac{10}{10}$ | $\frac{9}{10}$ | $\frac{10}{10}$ | $\frac{6}{10}$ | $\frac{3}{10}$ |
| 114 | $\frac{10}{10}$ | $\frac{10}{10}$ | $\frac{10}{10}$ | $\frac{4}{10}$ | $\frac{1}{10}$ |
| 3166 | $\frac{10}{10}$ | $\frac{0}{10}$ | $\frac{0}{10}$ | $\frac{0}{10}$ | $\frac{0}{10}$ |
| B1211 | $\frac{10}{10}$ | $\frac{10}{10}$ | $\frac{10}{10}$ | $\frac{8}{10}$ | $\frac{3}{10}$ |

Numerator indicates the number of mice dead.
Denominator indicates the number of mice injected.

TABLE 4.—MORTALITY RESULTS IN MICE WITH 4 STRAINS OF *C. tropicalis*

For comparison the lethality for mice of 5 strains of *C. albicans* representing both extremes and intermediate virulence are shown in table 5.

It is quite apparent from these results that most strains of *C. tropicalis* are quite pathogenic for mice, but not for rabbits. *C. tropicalis* is frequently isolated from human sources, but seldom is a medical problem. This suggests that man's susceptibility to infections by this yeast is more parallel to that of the rabbit, or possibly that more human infections occur due to this organism and that the proper identification is not made.

*In vivo* multiplication rates for 2 strains of *C. albicans* are shown in figs. 1 and 2.

| *Strain No.* | *No. of Cells Injected* | | | | |
|---|---|---|---|---|---|
| | $10^7$ | $10^6$ | $10^5$ | $10^4$ | $10^3$ |
| 857 | 5/5 | 0/5 | 0/5 | 0/5 | * |
| B311 | 5/5 | 5/5 | 5/5 | 2/5 | 0/5 |
| 792 | 5/5 | 5/5 | 5/5 | 2/5 | 0/5 |
| 3162 | 5/5 | 5/5 | 5/5 | 4/5 | 1/5 |
| 275 | 5/5 | 5/5 | 2/5 | 1/5 | 0/5 |

Numerator indicates the number of mice dead.
Denominator indicates the number of mice injected.
* Omitted.

TABLE 5.—MORTALITY RESULTS IN MICE WITH 5 STRAINS OF *C. albicans*.

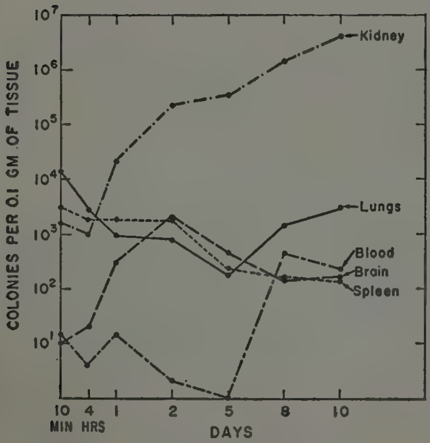

Figure 1.—Multiplication rate in mice of *C. albicans* strain B311.

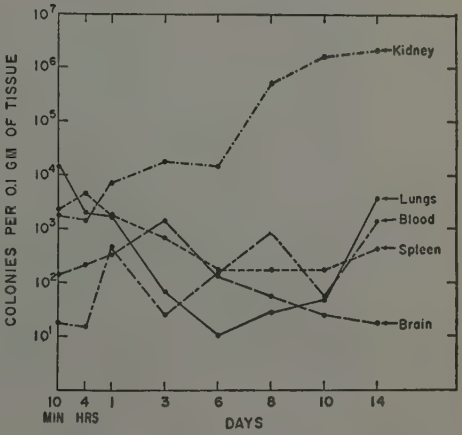

Figure 2.—Multiplication rate in mice of *C. albicans* strain 792.

These results show that most of the yeast cell or mycelial propagation occurs in the kidney. Louria (1960) had previously reported similar evidence.

Growth rates in mice for 3 strains of *C. tropicalis* are presented in figs. 3, 4, and 5. It is apparent that there was some strain variation in the time required to produce terminal disease, but multiplication in kidney tissue appeared to be similar to that of the *C. albicans* strains. An interesting difference, however, between the 2 species was the response of *C. tropicalis* in the brain. The initial growth, climaxed 2–4 days after injection, with a subsequent fall of viable yeast cell count, was observed with all 3 strains. Sections of brain tissue made from mice infected for 2 and 8 days confirmed the cultural observations. Fig. 6 shows

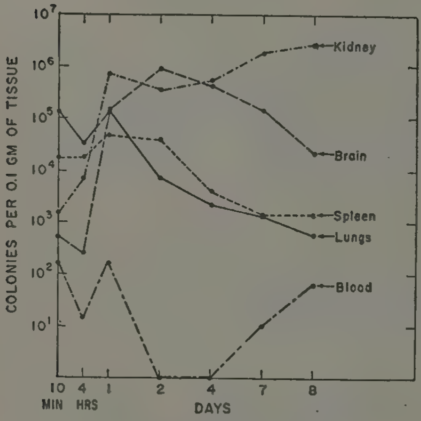

Figure 3.—Multiplication rate in mice of *C. tropicalis* strain 48.

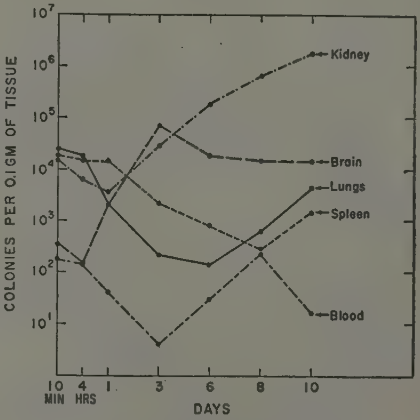

Figure 4.—Multiplication rate in mice of *C. tropicalis* strain 20.

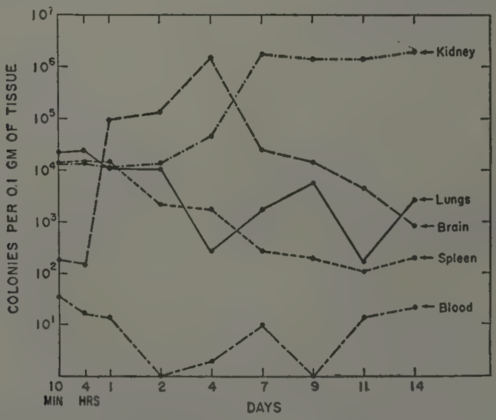

Figure 5.—Multiplication rate in mice of *C. tropicalis* strain 114.

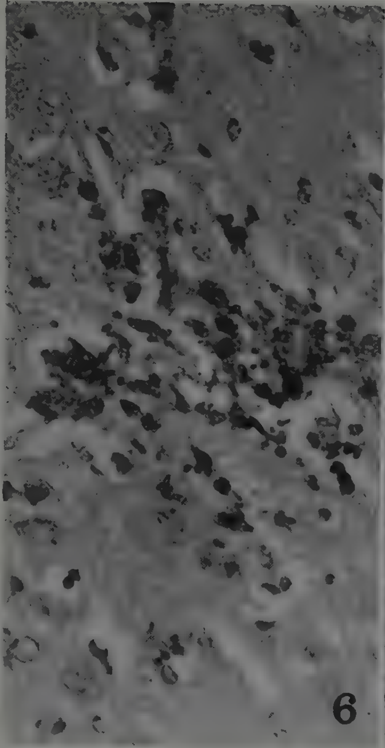

Figure 6.—Brain lesion due to *C. tropicalis* 2 days after infection × 400.

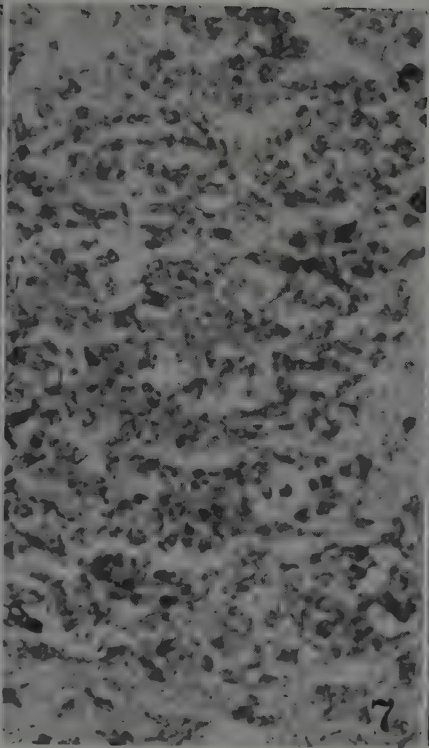

Figure 7.—Brain lesion due to *C. tropicalis* 8 days after infection × 515.

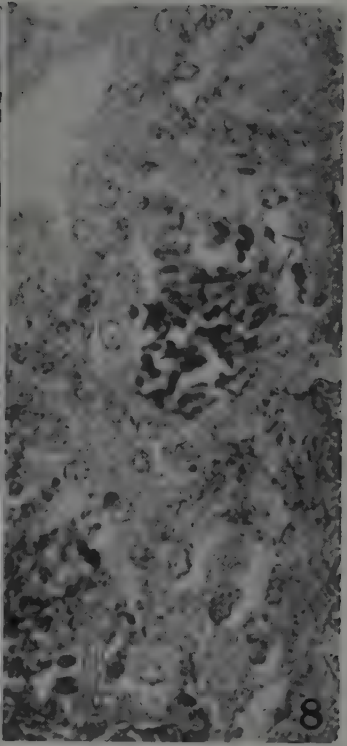

Figure 8.—Kidney lesion due to *C. tropicalis* 2 days after infection × 515.

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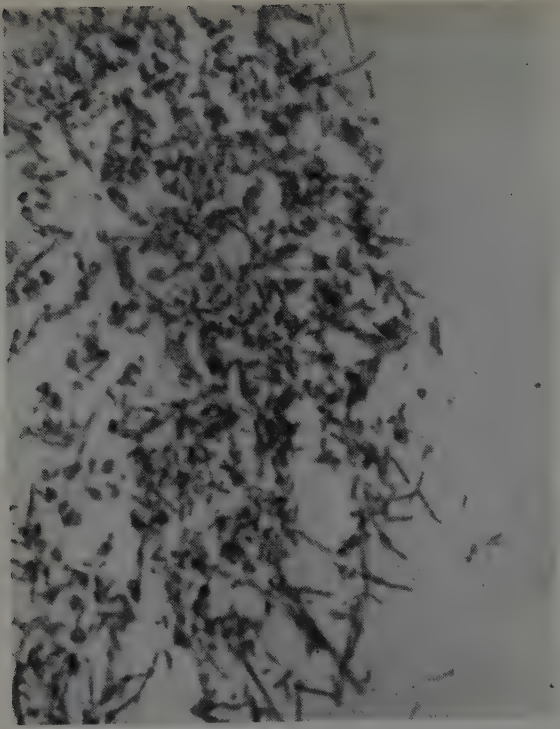
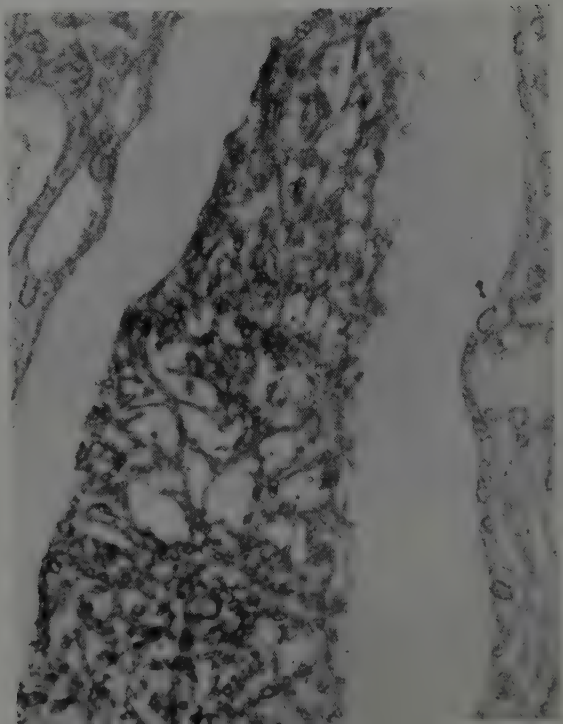


Figure 9.—Kidney lesions due to *C. tropicalis* 8 days after infection × 515.

Figure 10.—Kidney lesion due to *C. albicans* 8 days after infection × 515.



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a focal lesion in which there are a number of yeast cells, and only a few histiocytes. Lesions in the brain were numerous at this time and easy to find. Fig. 7 indicates the appearance of the lesion 8 days after infection, and when the surviving mice were in the final stages of disease. At this time there was a much greater cellular response and with only a few yeast cells present. These observations represent an unusual host reaction in the brain and suggests that the cellular defence was able to destroy, after the initial increase, part or most of the yeast cell population.

Figs. 8 & 9 demonstrate the appearance of kidney lesions due to *C. tropicalis* 2 and 8 days after injection, respectively.

Fig. 10 shows the appearance of a kidney lesion, due to *C. albicans*, 8 days after infection, and indicates that there has been a large amount of tissue destruction.

The data presented here indicate that, as a group, the strains of *C. albicans* showed slightly more mouse virulence than the strains of *C. tropicalis*. Individually, however, a few of the strains of *C. tropicalis* were more virulent than some strains of *C. albicans*. Mouse pathogenicity therefore could not be used in the laboratory as a means of separating these 2 species. Rabbit pathogenicity was a reliable means of differentiation.

Another question which merits discussion is the identity, or the criteria used for the identification, of the strains of *C. tropicalis*. All of these isolates rapidly fermented glucose, maltose, sucrose, and galactose, and utilized these carbohydrates as a sole source of carbon. Ethanol was also utilized as a carbon source. None of them utilized, or fermented lactose, and none produced chlamydospores on corn meal agar or rice Tween 80 agar. These characteristics, together with the lack of or a very low degree of rabbit pathogenicity indicate the correct identification.

## Résumé

La plupart des races de *Candida tropicalis* possèdent une virulence considérable pour les souris. Comparées en groupe, les races de *C. tropicalis* étaient d'une virulence inférieure, ainsi que les races étudiées de *C. albicans,* et montraient peu de pathogénicité, ou presque aucune, pour les lapins.

*In vivo* les taux de multiplication montraient que pour les deux espéces la plupart de la prolifèration de loge de levure, ou d'aggregat de loge de levure, se produisait dans des tissus rénaux. *C. tropicalis* en différait en ce qu'une croissance de loge de levure se manifestait tôt dans des cerveaux de souris infectées, 2 à 4 jours après injection, accompagnée d'une décroissance consécutive de loges de levure viables. Des sections histologiques de tissu de cerveau ont confirmé ces observations culturelles.

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# MONILIASIS IN PARTRIDGES (*PERDIX PERDIX*)

I. F. Keymer and P. K. C. Austwick

*Ministry of Agriculture, Fisheries and Food,*
*Central Veterinary Laboratory, Weybridge.*

An outbreak of moniliasis (candidiasis) in artificially reared partridges was associated with heavy losses. The clinical signs were mainly inco-ordination, but lesions were observed on the tongue, in the crop and on the digits of the affected birds. *Candida albicans* was isolated from all lesions and also in quantity from the environment of the birds. Histological examination showed the presence of the fungus in the lesions without any marked reaction. Treatment with oxytetracycline hydrochloride and phenylmercuric dinaphthylmethane disulphonate was ineffective but the outbreak cleared up when nystatin was added to the drinking water.

Moniliasis (*Candida albicans* infection) occurs in domestic poultry, especially turkeys, but the disease has also been recorded in various game birds. Jungherr (1933) reported the infection in artificially hatched pheasants (*Phasianus colchicus*) and in ruffed grouse (*Bonasa umbellus*) in the United States of America, and Kuprowski (1956) recorded the infection in capercaillie (*Tetrao urogallus*) in Poland. The disease does not appear to have been previously recorded in partridges, and this paper describes an outbreak in which heavy losses occurred with the recovery of *C. albicans* in quantity from the environment.

## HISTORY

The outbreak commenced in the last week of July 1958 amongst artificially reared partridges on a gamebird farm in Buckinghamshire. Losses began on the same day in 2 groups of birds situated a mile apart, 200 birds being involved.

Three or 4 birds died each day during the first 15 days of the outbreak, before any treatment was given. A 10 day course of treatment with oxytetracycline hydrochloride* and phenylmercuric dinaphthylmethane disulphonate† was then begun and during this period a further 24 birds died. Two days later, nystatin§ therapy was commenced and only 2 more deaths were subsequently reported. Although detailed records were not kept, the estimated total loss was nearly 50 %.

The owner had had considerable experience of rearing pheasants on a large scale, but until the summer of 1958 partridges had only been raised in small numbers by giving them preferential treatment. At the beginning of the breeding season in 1958, however, 200 partridges had been successfully reared and the same system of management was used for the later hatches which developed moniliasis.

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* "Terramycin". † "Penatrone". § "Mycostatin".

### *Management*

The partridge eggs were incubated by bantams on farm A. When the young were a few days old they were transferred to a lawn and kept in houses and runs which had no floor, the birds being in direct contact with the grass. Each house was provided with a bright-emitter infra-red lamp suspended about 2½ ft. from the ground and about a dozen birds were housed in each unit, there being no overcrowding. The units were moved on to fresh grass every 4 to 5 days and no birds had been reared previously on the lawn. In spite of a considerable amount of rain prior to the outbreak no mud formed in the runs.

Food was provided in metal containers, insoluble grit in metal trays and water was obtained from small automatic metal drinking fountains, all of which were kept clean. Fresh tap water was supplied daily.

At approximately 6 weeks of age the birds were transferred from the lawn pens to the rearing field on farm B about 1 mile away, where they were kept in open wire netting runs with a hen coop for shelter. The runs were arranged in long rows at least 4 ft. apart and were moved frequently. The field which was a ley pasture sloped slightly, the drainage being good and no poultry had been kept on it for at least 20 years.

### *Feeding*

The exact constituents of the diet were not known, but from the information available, and in the absence of knowledge on the exact nutrient requirements of partridges, the food appeared to be satisfactory for growing birds up to 2½ weeks of age. At first the ration consisted primarily of pheasant poult starter pellets containing, in addition to carbohydrate (chiefly in the form of Sussex ground oats), 28 % protein, 5 % oils, 4 % fibre, 3 % yeast powder, 2–5 % minerals, 0·5 % sodium chloride, and 2 % cod liver oil. At 2½ weeks of age, the diet was gradually changed to one which apparently contained less protein. At this stage there also appeared to be a reduction in vitamins of the B complex, owing to the withdrawal of the yeast powder.

Until 6 weeks of age (*i.e.,* whilst at farm A) greenfood was provided in the form of lettuce, in addition to that obtainable from the lawn vegetation. After a few weeks the diet was gradually changed again to the final rearing ration, the proportion of protein being further reduced and that of carbohydrate increased by approximately two thirds. No antibiotics, coccidiostats or drugs of any kind were incorporated in the food.

### *Clinical Signs*

The owner stated that young partridges varying in age from a few days to 12 weeks were affected. The first sign was an unsteady gait, which was only noticeable when running, the birds otherwise appearing normal. A short while before death, an affected bird would be found lying on its side, unable to stand, but usually with its head and neck held erect. The bird chirped and generally appeared bright until death was imminent. When the farm was visited, none of the partridges was in an advanced stage of the disease but 1 specimen showing marked inco-ordination and staggering gait, was taken back to the laboratory for observation. Death occurred 36 hours later, during the night; no further deterioration having been seen before death.

The farm was visited 24 days after losses had commenced, and some of the partridges at farm A were noted to be badly feathered. Secondary wing feathers showed some loss of pigmentation, fraying and slight curling, giving the birds a rather ragged appearance. Only a few of these birds developed moniliasis, and none affected in this way were received for autopsy. In each of the 3 groups of birds kept at farm A, there were 1 or 2 showing inco-ordination, which was barely noticeable unless the birds were disturbed and buffetted by their healthy companions.

At the time of the visit, the partridges on farm B were all about 6 weeks old, and few showed clinical signs although some feather pecking was evident. Close examination of the birds, however, was not possible owing to their nervous temperament.

### *Post-Mortem Lesions*

A total of 13 specimens was received for autopsy. All were in good condition. Three birds showed scabby encrustations at the commissures of the mouth and also slight lesions on the tongue and palate. The same birds also showed slight dermatitis on the plantar aspects of the toes characterised by shallow circumscribed and brownish lesions overlying the phalangeal joints (fig. 1).

The only macroscopic lesions seen internally involved the crop and proventriculus and in 3 specimens no obvious lesions were present. In 9 the crop showed acute lesions of moniliasis characterised by a greyish white flocculent exudate loosely adherent to the mucous membrane (fig. 2). In 1 bird there were similar lesions in the proventriculus, although the crop appeared normal.

### *Mycological Examination*

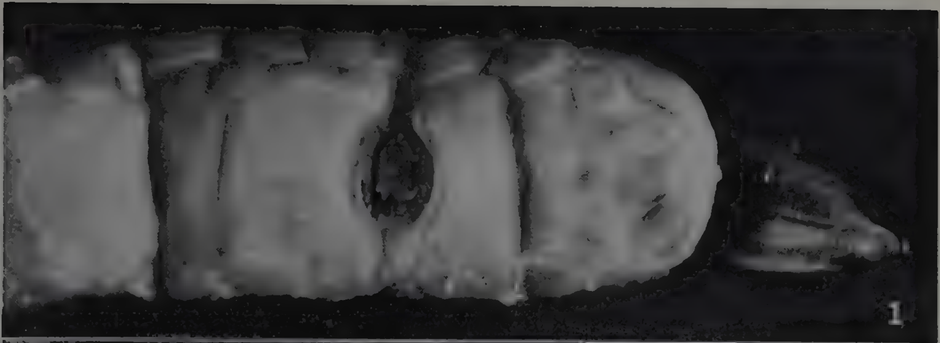
Direct microscopical examination of the scabby material from the commissures of the mouth, the whitish mucoid patches on the tongue and palate, and the greyish flocculent exudate from the crop and proventriculus, revealed abundant pseudo-hyphae and yeast cells. Small pieces of material from the foot lesions were mounted in 20 % potassium hydroxide, gently warmed and then crushed, before examination. Numerous pseudo-hyphae could be seen penetrating the keratinised tissue.

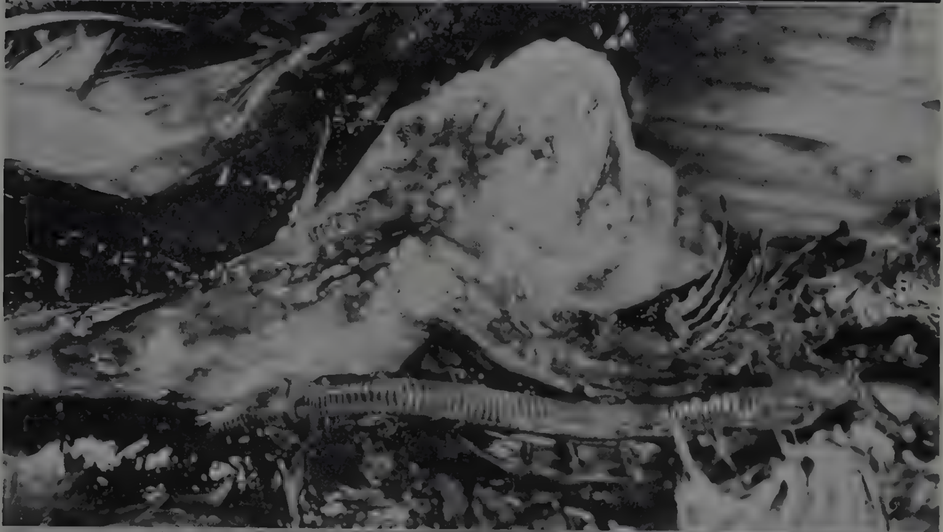
Exudate from the crop epithelium of 5 birds was cultured by streaking on 2 % malt extract agar plates containing a supplement of 20 units penicillin and 40 units streptomycin/ml. Foot lesions were examined by dissecting out small pieces of the stratum corneum and distributing them on the same medium. Incubation was at 37° C. Samples of vegetation and other material were also examined by direct plating and by dilution plating.

## RESULTS

*Candida albicans* (Robin) Berkhout was isolated in quantity from all 5 crops and in addition other yeasts were isolated, including *Saccharomyces tellustris* van der Walt (the perfect state of *Candida bovina* van Uden) from 2 crops.

A profuse growth of *C. albicans* was obtained from the foot lesions of 1 bird





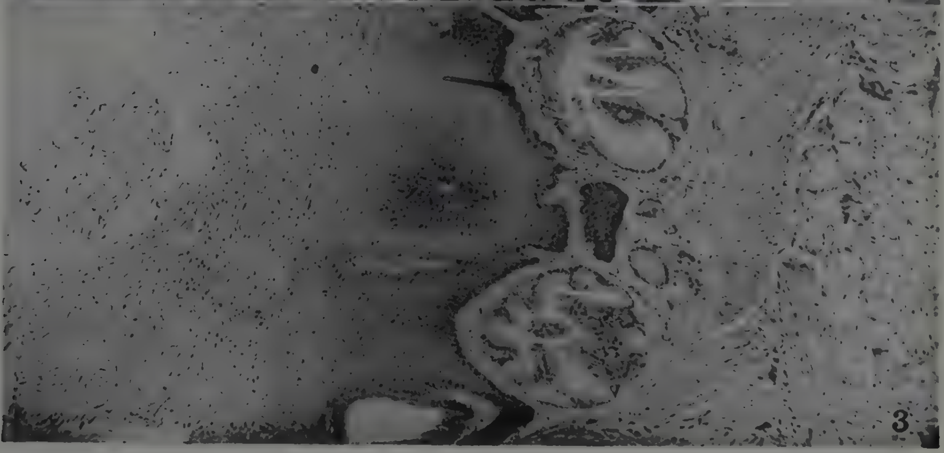


Figure 1.—Lesion on digit of a partridge, associated with *Candida albicans* × 10½.

Figure 2.—Opened crop showing typical lesions of moniliasis × 2.

Figure 3.—Section of crop stained with haematoxylin and eosin, showing extensive superficial accumulation of epithelial cells × 70.

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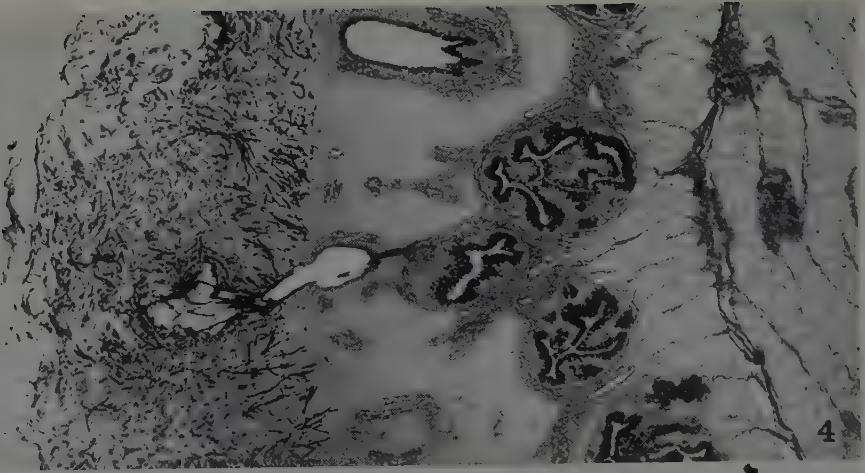

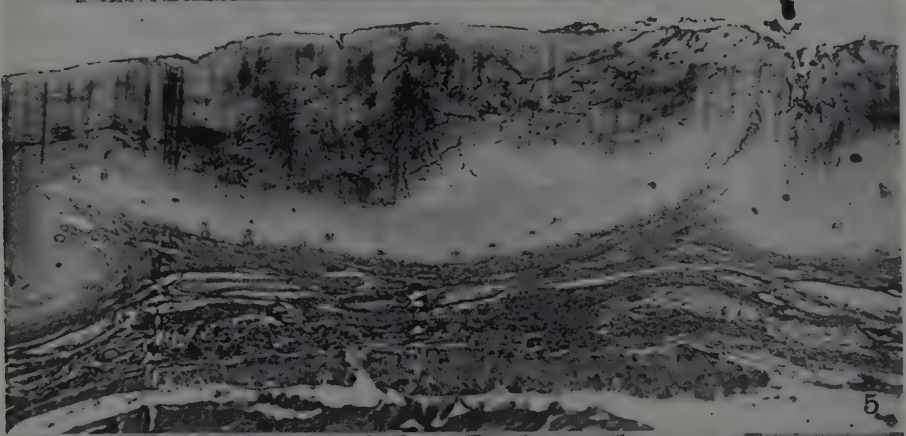

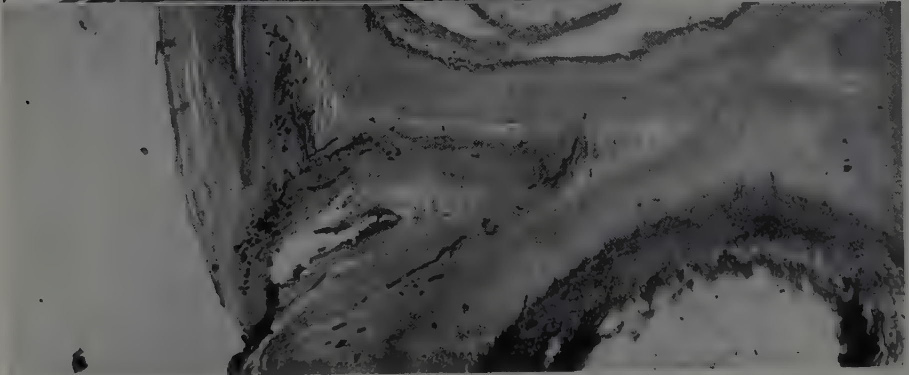

Figure 4.—Section of crop stained with the periodic acid-Schiff technique, showing numerous pseudo-hyphae penetrating the stratified squamous epithelium × 70.

Figure 5. Longitudinal section of the plantar aspect of the foot stained with the periodic acid-Schiff technique, showing thickening of the stratum corneum and invasion by pseudo-hyphae × 70.

Figure 6. Longitudinal section of the dorsal surface of a toe through a fold between two scale plates, showing deep penetration of pseudo-hyphae × 70.

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together with an unknown yeast, and from another foot a further unknown yeast was isolated, but no *C. albicans*. In 1 bird, attempts to isolate *C. albicans* from the brain, liver and kidneys were unsuccessful although the yeast was isolated from the intestine.

### *Examination for other Pathogens*

In all birds routine bacteriological examination was carried out, but in none was there any evidence of bacterial disease, nor were the lesions suggestive of the presence of a specific virus infection. With the exception of 1 specimen, which harboured a moderately severe infection of duodenal coccidiosis, no parasitic disease was diagnosed.

### *Isolation of* C. albicans *from the Environment*

In the lawn pen on farm A which housed the youngest chicks, the vegetation consisted primarily of various grasses (*Agrostis* and *Festuca* spp.), yarrow (*Achillea millefolium* L.), small patches of clover (*Trifolium* spp.), daisies (*Bellis perennis* L.) and a small number of other rosette plants such as plantains (*Plantago major* L. and *P. lanceolata* L.) and dandelions (*Taraxacum officinale* Weber). Dilution plating of lawn samples yielded very large numbers of *C. albicans* cells estimated at about 10,000 cells/g. (undried). This finding was repeated with a second sample. From food and water in this pen no yeasts were isolated, but from the grit 2 colonies of *C. albicans* were obtained. No yeasts were isolated from a sack which was partly covering 1 run.

On farm B in a pen containing birds which were 7 weeks old, *C. albicans* was also isolated from the vegetation (mainly cock's-foot (*Dactylis glomerata L.*)) clover, ox-eye daisies (*Chrysanthemum leucanthemum L.*) and docks (*Rumex* spp.) but only in small numbers and not from the grit or the water. In another pen, however, both the vegetation and the drinking water harboured large numbers of 2 different yeasts, neither of which was *C. albicans*.

Several days later further samples were collected. One, from the lawn grass on farm A in an area away from the partridge pens, gave a fairly large number of colonies of *C. albicans*, but from the other sample of meadow grass on farm B, also from an area away from the pens, no yeasts were isolated.

A second visit was made to the farm on the 28th July, 1959, 1 yr. after the outbreak, but the lawn was not being used for partridge rearing and a different part of the field from the previous year was used at farm B. No case of moniliasis had been diagnosed in birds raised that season, but because it was thought that *C. albicans* might be recovered again from the lawn on farm A, samples were taken from various places, both inside and outside the sites of the previous year's pens.

Samples taken from the lawn on farm A at the site of a previous year's pen revealed no yeast isolates. From an area some distance away which had not been used, *C. albicans* was isolated in small numbers, together with other yeasts. Further yeasts were recovered from a pheasant pen on farm B, but none from other sites in this field, including the partridge pens. None of these yeasts, however, proved to be *C. albicans*.

### *Histology*

Tissues for histological examination were fixed in 10 % formol saline. Three staining methods were used for sections of the tongue, crop and digits, *viz.*: haematoxylin and eosin, periodic acid Schiff and Gomori-Gridley.

Sections of the brain (cerebrum and cerebellum) were stained with haematoxylin and eosin, by Romanes' technique, and with copper phthallocyanin. Organs examined histologically were taken from 2 birds which showed macroscopic lesions in the crops and on the feet. The same organs taken from healthy partridges were also examined for comparison.

*Tongue.* There was marked necrosis of the walls of the dorsal fissure associated with an accumulation of epithelial debris, which contained only very few yeast cells. No actual inflammatory reaction was present but little remained of the epithelium in this area. Pseudo-hyphae had penetrated into the corium surrounding the entoglossal cartilages and at one point the fungus had invaded the periphery of the cartilage.

*Crop.* The crops of 2 birds, from which *C. albicans* had been isolated, were examined histologically. In one (figs. 3, 4) there was an extensive superficial accumulation of epithelial cells, but virtually no inflammatory reaction, although a few eosinophils were present below the Malpighian layer. The stratified squamous epithelium was especially thick in the folds of the crop, and cushions of desquamating cells were present containing large numbers of pseudo-hyphae growing directly downwards towards the base of the epithelium. The outer layers of the stratum corneum were slightly necrotic showing loss of structure and a certain amount of vacuolation. The inner layers of the squamous epithelium into which the pseudo-hyphae did not penetrate, were quite unaltered and the mucus glands were also unaffected.

The other crop was comparatively mildly affected and the accumulation of epithelial debris on the surface of the squamous epithelium was much smaller. A few pseudo-hyphae were present in the epithelial debris which was lodged in the folds of the crop wall. There was far less degeneration of the outer layers of epithelium and no vacuolation. In both sections of the crop, the results obtained by staining with the periodic acid-Schiff technique were superior to the Gomori-Gridley method.

*Feet.* Sections of the foot lesions (fig. 5) showed areas of extensive thickening and invasion of the stratum corneum of the scales on the plantar surface of the toes with the growth of pseudo-hyphae parallel to the Malpighian layer but with little tissue reaction or necrosis. Towards the surface, individual yeast cells had budded off and the deep penetration of the fungus in this particular way may denote that the infection was relatively chronic. In the scales on the dorsal aspect of the toes, the fungus was invading the folds between 2 scale plates without apparently producing lesions (fig. 6). In most cases pseudo-hyphae extended under the posterior lip of the fold into the stratum corneum, but did not penetrate the Malpighian layer. Yeast cells seen in the epithelial debris in these sections may have been related to the other isolates obtained.

*Brain.* No definite lesions were found in sections of the cerebrum and cerebellum, although only a small number of sections was examined.

### *Treatment*

Fifteen days after losses started, some of the birds were treated with oxytetracycline hydrochloride but after a temporary improvement, there was a relapse. Another group was treated with phenylmercuric dinaphthylmethane disulphonate and some birds were given oxytetracycline hydrochloride in addition to this drug, but with no apparent improvement. In each case the drugs were administered in the drinking water. These treatments were stopped after 10 days and 2 days later nystatin therapy was commenced. The antibiotic was obtained in the form of a micronised powder which was partly soluble in water, the equivalent of 300,000 units of nystatin being mixed with 2 gallons of the drinking water. This medicated water was given for a period of 14 days. From the time the nystatin treatment was commenced, complete recovery occurred, with the exception of 2 birds which were found 'off their legs' by the owner, 3 days after the end of the treatment. These birds were killed and submitted for autopsy. No macroscopic lesions were present, although *C. albicans* was isolated from the crops of both birds.

Following this treatment the owner was advised to incorporate dried brewer's yeast in the food, in the proportion of 3 to 4 % of the total ration and he later reported that all survivors matured satisfactorily. It is not possible to draw any definite conclusions from the results of the above therapy, although it is worth noting that losses stopped when nystatin treatment was begun.

## Discussion

Apart from being apparently the first record of moniliasis in partridges, investigation of this outbreak has provided epidemiological evidence that *C. albicans* can grow and persist outside the animal body. This is indicated by the isolation of the yeast in quantity from the vegetation in the partridge pen but what is more interesting is its isolation from vegetation not contaminated by partridges. The fact that the yeast was isolated from the intestine also indicates that the organism is excreted in the droppings of infected birds and this may have been partly responsible for the heavy contamination of the vegetation in the pen containing affected birds. It seems possible that under certain circumstances *C. albicans* may occur naturally on some types of vegetation in the summer, and that the presence of numerous susceptible birds can result in an increase of its population so that the yeast has a large inoculum potential.

There appear to be only 6 records of the isolation of *C. albicans* from outside the animal body and it does not seem to have been recovered in great quantity on any of these occasions. Di Menna (1958) was not certain that it is a characteristic yeast of grassland, but now it seems possible that if a systematic search was made at the right time of the year the yeast might well be found more frequently.

The occurrence of *C. albicans* in foot lesions of birds does not appear to have been reported previously, although subsequent observations (Carnaghan & Austwick, 1959) have shown that similar, although more severe lesions may sometimes occur in turkeys affected with moniliasis of the crop. It seems unlikely, however, that *C. albicans* is the primary cause of these lesions, the yeast probably being a secondary invader. The lesions closely resemble dietetic dermatitis described in chicks and turkeys associated with pantothenic acid, biotin, riboflavin and niacin deficiencies (Hinshaw, 1959; Scott & Norris, 1959). Blaxland & Fincham (1950) described similar lesions on the feet in turkey poults affected with moniliasis but whether or not these were associated with *C. albicans* infection is not known. In some groups of these turkeys feather pecking occurred, and this was also evident in certain of the affected partridges in this outbreak.

It is now generally accepted, as the result of investigations into previous cases of moniliasis in turkeys (Hinshaw, 1934; Blaxland & Fincham, 1950; Blaxland & Markson, 1954), that predisposing causes are important in precipitating an outbreak. Blaxland (1951), for example, stressed the importance of unhygienic surroundings, overcrowding and dietetic deficiencies. In this outbreak, however, a dietary deficiency seemed to be the only possible predisposing factor and even then, as far as vitamins of the B group were concerned the diet only appeared to be deficient for the partridge chicks over 2½ weeks of age. According to the owner a few younger birds had been affected with moniliasis. The fact that birds of all ages on different diets became affected at exactly the same time, does not indicate that the primary cause was a nutritional deficiency, unless this was brought about in some way by the presence of *C. albicans*. Blaxland and Fincham (1950) suggested that 'the yeast in the crop may utilise vitamin B in the food or prevent its absorption'.

Unlike turkeys, little is known of the nutrient requirement of partridges and it is interesting to note that live food, mainly in the form of small soft bodied insects constitute over 90% of the diet of wild partridges up to 14 days of age (I.C.I. Game Services Advisory Booklet No. 18, 1954). At the age of 2–3 weeks animal food primarily in the form of ants and their pupae was found to comprise 52·5%. It is not until over 3 weeks of age that plant food becomes the main constituent of the diet, *i.e.*, 96·7%. There is little doubt that these artificially reared birds obtained only an insignificant amount of live food in the restricted area of their pens and their diet may therefore have been deficient in some unknown requirements.

Another confusing aspect of this disease is the reason for the clinical signs shown by affected birds, particularly as different ones were described by the owners of hand-reared partridges in two further outbreaks of moniliasis in other districts, during July and Aug. 1959. In 1 of these, the birds were said to have shown retraction or twisting of the head and neck. Those in the other outbreak simply ceased to grow, showed ruffled plumage (especially around the head and neck), became weak and died. These outbreaks appear to represent acute and chronic forms of the disease respectively, and are similar to those described in turkey poults by Blaxland & Fincham (1950).

It is difficult to believe that the presence of *C. albicans* associated with the lesions described in this paper could be entirely responsible for the clinical signs and death. Assuming that no undiagnosed disease was present there appear to be only two possible explanations; either the yeast is capable of producing a toxaemia as suggested by Adriano & Schwarz (1955), or the clinical signs are due to a nutritional deficiency brought about by the presence of *C. albicans* in the alimentary tract. Salvin (1952) actually demonstrated an endotoxin in *C. albicans* but Blaxland & Markson (1954), as the result of experiments on the transmission of *C. albicans* to turkey poults, found nothing to suggest the production of a toxaemia by the yeast, although they were of the opinion that the infection 'consisted of something more than the mere presence of the organisms in the tissues'.

As the result of the investigations carried out in this outbreak and the findings of Blaxland & Markson and of Salvin, it would seem that a study of both the production of a toxaemia by *C. albicans* and the association of the yeast with vitamin deficiencies especially of the B group in partridges and turkeys might be of interest.

## RÉSUMÉ

Une épizootie de muguet (*Candida albicans*) dans un élévage de perdrix fut cause d'une mortalité élévée. La maladie était characterisée cliniquement surtout par inco-ordination; en plus, la langue, les doigts et la jabot des oiseaux malades portaient des lesions. On a isolée *Candida albicans* de toutes lesions, du gravier, de l'herbage. L'examen histologique a decelé le champignon dans des lesions qui ne presentaient aucune reaction. Le traitement par oxytetracycline hydrochloride et 'phenylmercuric dinaphthylmethane disulphonate' a donné des resultats negatifs mais on réussit à éteindre l'epizootie en ajoutant du nystatin à l'eau à boire.

## ACKNOWLEDGEMENTS

We are indebted to the owner of the partridges for his kind co-operation; to E. R. Squibb & Sons Ltd., for assistance in the use of nystatin; to Mr. E. Clements for photography, and to Mrs. J. D. Cartner for examining the yeast isolates.

Dr. A. M. Lucas of East Lansing, Michigan, U.S.A. was especially helpful in kindly providing references to published work on the histology of normal avian tissues and we also wish to thank Messrs. R. B. A. Carnaghan and L. M. Markson for help with the histopathology.

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# STUDIES OF THE INVASIVE, MYCELIAL FORM OF *CANDIDA ALBICANS*

G. A. Gresham and C. H. Whittle

*Department of Pathology and Department of Medicine,*
*The University, Cambridge.*

It is shown experimentally that iso-nicotinic acid hydrazide inhibits mycelial production by *Candida albicans in vitro* and *in vivo*. Despite this *C. albicans* given *i.v.* may still cause death in rabbits unless a loading dose of I.N.A.H. is given previous to the *C. albicans* injection and *i.p.* dosage is continued daily for 7 days. Animals protected in this way, nevertheless show small lesions in their kidneys when killed 18 days later. These lesions contain a small number of blastospores and short hyphae and resemble the pyelonephritis-like lesions in the kidneys of animals given sub-lethal doses of *C. albicans*.

By fluorescent microscopy, and by special staining methods, it was shown that ribo-nucleic acid is present throughout the entire length of *C. albicans* hyphae and is concentrated at the septa and hyphal tip. Since this is also seen in mycelial fungi such as *Aspergillus fumigatus* it supports the view that *C. albicans* forms true mycelium and suggests that the mycelial formation may help the fungus to invade tissues.

The precise way in which a micro-organism may injure host tissue is often obscure. *Candida albicans* in inflammatory lesions of the skin and mucosae in man is predominantly mycelial in form (Whittle, Moffatt & Davis, 1959; Whittle & Gresham, 1960a, 1960b), the abundance of mycelium affording some index of the severity of the inflammation. Studies of experimental moniliasis in the rabbit (Gresham & Burns, 1960) have confirmed the fact that the mycelial phase of the fungus is the invasive form and have indicated how such mycelium may cause tissue damage.

This paper is concerned with the significance of the effects of iso-nicotinic acid hydrazide (I.N.A.H.) on *C. albicans* in the rabbit and *in vitro* (Pesce de Ruiz Holdago, 1956). The experiments were designed to elucidate further the mode of disease production by the fungus.

## METHODS

*Culture: C. albicans* was grown in potato water (Langeron & Talice, 1932) with and without the addition of 0·1 % I.N.A.H. The resulting growth forms were studied by conventional methods of staining and by fluorescence microscopy with fluorochrome acridine orange which causes ribonucleic acid (R.N.A.) to fluoresce red and desoxyribonucleic acid (D.N.A.) to fluoresce green (Joshi & Korgaonkor, 1959).

*Animal inoculation:* Four groups of 6 rabbits, av. wt. 1·8 Kg., were inoculated *i.v.* with 1 ml. of a freshly prepared suspension of *C. albicans* in physiological saline ($500 \times 10^6$ cells/ml.). I.N.A.H. was given to the animals in each group as follows : (A) control group, no I.N.A.H.; (B) 10 mg. I.N.A.H. *i.v.* at time of inoculation; (C)

10 m.g. I.N.A.H. *i.v.* at time of inoculation followed by daily doses of 10 mg. *i.p.*; (D) 10 mg. I.N.A.H. *i.p.* on day before inoculation followed by daily *i.p.* doses of 10 mg. for 7 days.

## RESULTS

The formation of mycelium (fig. 1a) by *C. albicans* when grown in potato water was completely inhibited by the addition of 0·1 % I.N.A.H. (fig. 1b).

In the animal experiments the rabbits in groups A and B died in 3 days and there was no noticeable difference in the behaviour of animals in the control group A and those in group B. The rabbits in group C also died in an average of 3 days. However, there were gross differences in the kidneys of the animals in this group and those of the control group. The kidneys of the rabbits in the control group were greatly swollen and oedematous with many large white lesions (fig. 2a); those of rabbits in group C were smaller and the lesions smaller in size and fewer in number (fig. 2b).

The animals in group D, which had received the *i.p.* prophylactic dose of I.N.A.H. on the day before inoculation with *C. albicans* and a further 7 daily doses of I.N.A.H., survived. They remained well and were killed 18 days later. However, their kidneys were not normal and showed scanty small wedge-shaped lesions like pyelonephritis, with abundant inflammatory reaction (fig. 3).

Histological examinations of the kidneys of the various groups showed that in the control group A mycelium was present in abundance (fig. 4a). In groups B and C blastospores and short hyphae predominated (fig. 4b) and in group D the renal lesions showed very scanty fungal elements consisting of a few blastospores and short hyphae, insufficient to illustrate satisfactorily by photomicrographs.

## DISCUSSION

Mycelial formation by *C. albicans* was inhibited by the presence of I.N.A.H. both *in vitro*, in potato water, and *in vivo* when lethal doses of *C. albicans* were given *i.v.* to rabbits : though neither a single dose of 10 mg. of I.N.A.H. given *i.v.* at the same time as the infecting dose, nor this followed by daily *i.p.* doses of 10 mg. I.N.A.H., prevented the fatal outcome which occurred in about 3 days. However, those animals, which were given an additional loading dose of 10 mg. of I.N.A.H. *i.p.*, and were subsequently given daily I.N.A.H. *i.p.* for seven days, survived and when killed eighteen days later they showed definite lesions with scanty fungus in them.

*C. albicans* can therefore produce a fatal infection in rabbits even when mycelial production is impaired. Blastospores, by reason of their size and ability to plug small blood vessels in the kidney, cannot be responsible because the blastospores of *C. parapsilosis* of comparable size, given in comparable doses, do not kill a rabbit. Attention is thereby switched to the possibility in the pathogenic *C. albicans* of a capsular aggressin which is probably cytotoxic for vascular endothelium.

The fact that only those rabbits survived which were given a dose of I.N.A.H.

one day before the infecting dose of *C. albicans* seems to us of some importance. The lesions resembled those seen in animals given sub-lethal *i.v.* doses of *C. albicans* (Gresham & Burns 1960).

The I.N.A.H. may act in two distinct ways. It can certainly inhibit mycelial formation though this does not necessarily prevent a fatal outcome : it could also kill off, or inhibit, a sufficiently large number of the blastospores injected and reduce the effective dose, so that the infection is not lethal as in group D.

There is no doubt that the mycelial form of *C. albicans* is that most often found in preparations made from lesions in man and animals. It is, furthermore, an actively growing stage of the fungus as illustrated by the abundant production of ribo-nucleic acid in the mycelial form. The R.N.A. can be seen as metachromatic clumps, with methylene or toluidene blue staining (fig. 5) or as bright red fluorescent material when stained with acridine orange. The R.N.A. is located not only at the hyphal tip but also at the septa indicating an active production of the substance along the entire length of the hypha. This appearance is seen also in true mycelial fungi such as *Aspergillus fumigatus* (fig. 6) and lends support to the view that *C. albicans* produces a mycelium proper and not pseudo-mycelium as some would prefer to call it. This is no mere academic point since a hyphal thread implies functional or metabolic continuity along the entire length whereas pseudo-mycelium implies a chain of functionally disconnected units unable to extend from a zone rich in food material to one devoid of it. Such functional continuity could be advantageous in colonisation of tissue remote from the point of fungal entry into the tissue.

## RÉSUMÉ

Les résultats des expériences montrent que l'acide hydrazide iso-nicotinic empêche la production mycéliale par *C. albicans in vitro* et *in vivo*. Malgré cela *C. albicans* donné *i.v.* peut néanmoins tuer les lapins à moins qu'une dosification d' I.N.A.H. ne soit donnée avant l'injection de *C. albicans* et que cette

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## LEGENDS

Figure 1a.—*C. albicans* in potato water showing mycelium (Toluidene blue × 880).

Figure 1b.—*C. albicans* in potato water with 0·1% I.N.A.H. added : note blastospores with absence of mycelium (Toluidene blue × 880).

Figure 2a.—Whole kidney of rabbit, dying three days after *i.v.* injection of *C. albicans,* showing abundant gross white infective foci.

Figure 2b.—Less swollen kidney with fewer and smaller white foci in rabbit dying 3 days after *i.v.* injection of *C. albicans* and 10 mg. I.N.A.H.

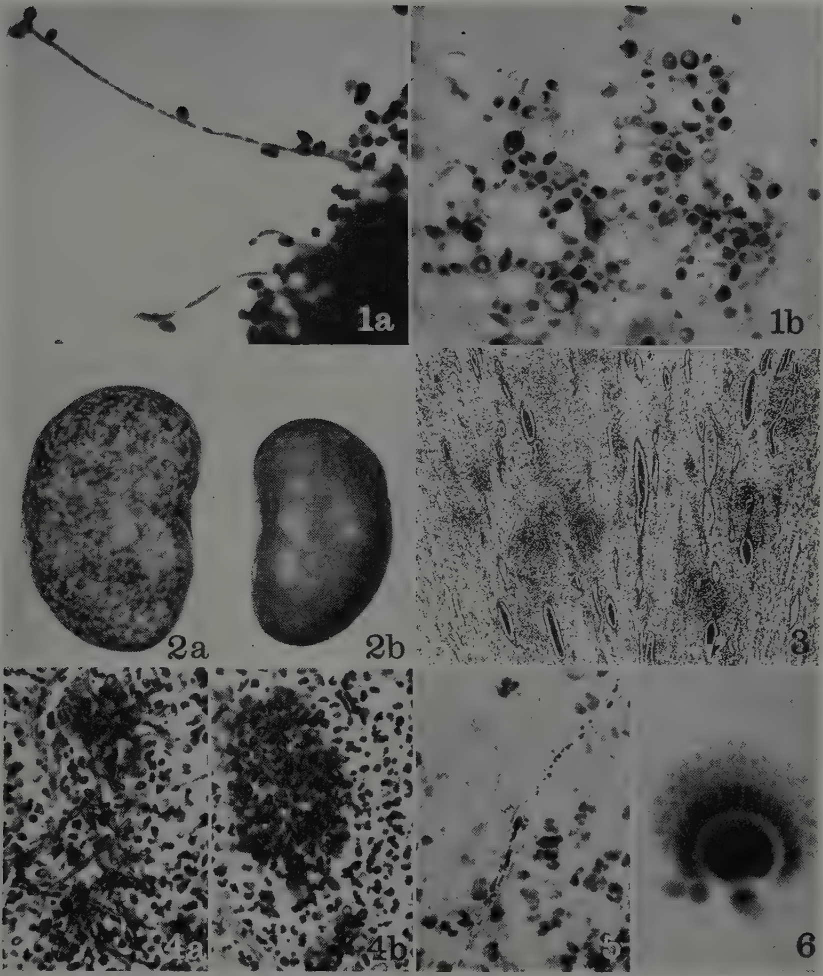
Figure 3.—Section of kidney of rabbit killed 18 days after *i.v.* injection of *C. albicans* and I.N.A.H. but also given a daily loading dose prior to and after the *C. albicans* was injected. It shows well defined inflammatory foci which contain, though these are not visible at this magnification, sparse hyphae and blastospores. (P.A.S. × 32).

Figure 4a.—Section of kidney of rabbit dying 3 days after *i.v. C. albicans* to show abundant mycelium (P.A.S. × 320).

Figure 4b.—Section of kidney of rabbit dying 3 days after *i.v.* of *C. albicans* with 10 mg. I.N.A.H. to show blastospores with little mycelium. (P.A.S. × 320).

Figure 5.—Mycelium of *C. albicans* in pus from human nail fold (perionychia) to show R.N.A. in clumps in mycelium, sometimes concentrated at septa. (Toluidene blue × 320).

Figure 6.—Conidiophore of *A. fumigatus* showing R.N.A. in tip (black area). This is reproduced from a fluorescent colour photomicrograph which shows the R.N.A. as red and the D.N.A .in the spores as green (Acridine orange × 1600).


1a
1b
2a
2b
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4a
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5
6

(*See* legends p. 32)

(*To face page* 32

dosification *i.p.* ne soit continuée journellement pendant sept jours. Même ainsi protégés, cependant, les lapins, si on les tue dix-huit jours plus tard, montrent de petites lésions rénales contenant quelques blastospores et de courtes hyphes et pareilles aux lésions de pyelonephritis observées dans des animaux auxquels l'on a donn' une dose sub-mortelle de *C. albicans*.

Le microscope fluorescent et l'emploi de methodes spéciales de coloration, ont révélé la présence de l'acide ribonucleic d'un bout à l'autre de l'hyphe, mais concentré aux extrémités. Cette présence qu'on rencontre dans des champignons mycéliaux tel *Aspergillus fumigatus*, soutient la conclusion que *C. albicans* produit du vrai mycélium et évoque la possibilité que cette formation mycéliale aide le champignon à envahir les tissus.

## ACKNOWLEDGEMENTS

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# REPRODUCTION AND PATHOGENICITY OF *CRYPTOCOCCUS NEOFORMANS*

F. Bergman

*University Department of Pathology,*
*General Hospital, Malmö, Sweden.*

Five strains of *Cryptococcus neoformans* were studied with regard to their reproduction on solid medium and virulence for mice. The reproduction, in terms of budding organisms, began to decrease within 48 hours of inoculation and no difference was found between cultures grown on 5 day old and 15 day old media.

No difference, in their virulence for mice, was found in the 5 strains and no increased virulence, as demonstrated by intracerebral injection of mice, resulted from 42–57 mouse passages with intervening 6–10 days' culture on artificial media.

It may now be regarded as established that animal passage enhances bacterial virulence and this is ascribed to selection of virulent mutants present in the original culture. The increase in virulence obtained varies in degree, not only from one type of micro-organism to another, but also between micro-organisms in the same group. For any comparison of the virulence of different strains in a given group of micro-organisms to be acceptable, the experiments must be carried out under uniform conditions, *e.g.*, the composition and age of the medium used must be standard because differences in the medium are capable of influencing the rate of growth of the micro-organisms.

As a link in an experimental investigation of the pathogenesis of cryptococcosis the growth rate of 5 different strains was determined in terms of the proportion of budding organisms. In addition the virulence of the 5 strains was compared by study of their lethal effect in mice and the effect on virulence for mice of a varying number of mouse passages with alternating culture on artificial medium was studied in 1 strain.

## Materials and Methods

The material consisted of 5 strains of *Cryptococcus neoformans* each of which came from a human case of cryptococcosis. Strains* (1) Malmo (2) No. 2224, (3) No. 2437 (4) No. 2507 were isolated from spinal fluid and (5) No. 2464 was cultured from lungs.

Cells from each strain were passed through 2 different series of adult albino mice with intervening culture on Sabouraud's dextrose agar containing penicillin and streptomycin. The animals received an *i.p.* injection of 0·5 ml. saline suspension of a 6–10 day old culture of *C. neoformans*, approx. $20 \times 10^6$ cells/injection. Each group consisted of 3 animals. In the first series surviving animals were killed 5 days and in the second series 10 days, after the injection. The animals were autopsied and intraabdominal swabs were cultured on Sabouraud's dextrose agar at room temperature. After 6–10 days' growth, new suspensions were prepared, a similar dose was injected into the next group of animals in the appropriate series and surviving animals were again killed 5 and 10 days after the injection. The numbers of passages through which the various strains were passed before they were tested for virulence are given in table 1.

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* The cultures of fungi (strain 2–5) were obtained through the courtesy of Dr. Norman F. Conant, Dept. of Bacteriology, Duke University School of Medicine, Durham, U.S.A.

| *Strain* | *Nos. of passages* | |
|---|---|---|
| | *Series 1* | *Series 2* |
| "Malmo" | 54 | 42 |
| No. 2224 | 56 | 35 |
| No. 2437 | 32 | 42 |
| No. 2507 | 57 | 41 |
| No. 2464 | 55 | 42 |

TABLE 1.—NUMBERS OF PASSAGES THROUGH WHICH THE 5 DIFFERENT STRAINS OF *C. neoformans* WERE CARRIED.

Determinations were made of the growth rate of the original cultures and of the subcultures obtained after alternating mouse and medium passages in both the 5 and 10 day series. From the original cultures of the 5 strains, which had been maintained for several years on artificial medium, cells from a 6-day old subculture were suspended in physiological saline and the suspension was cultured on Sabouraud's dextrose agar. At the same time swabs from the abdomens of the mice in series 1 and 2 were cultured on the same medium. To study the effect of the age of the medium

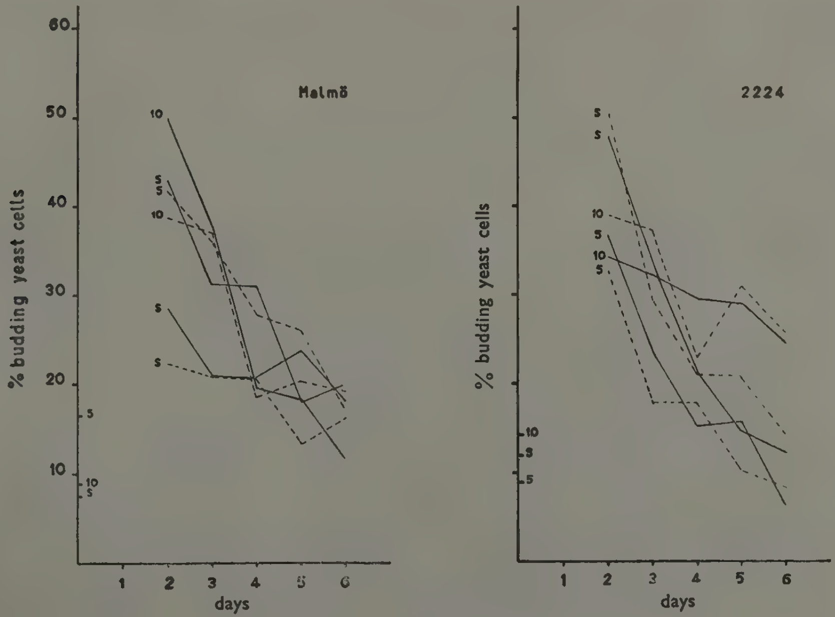

Figure 1. (*See* legend p. 36)

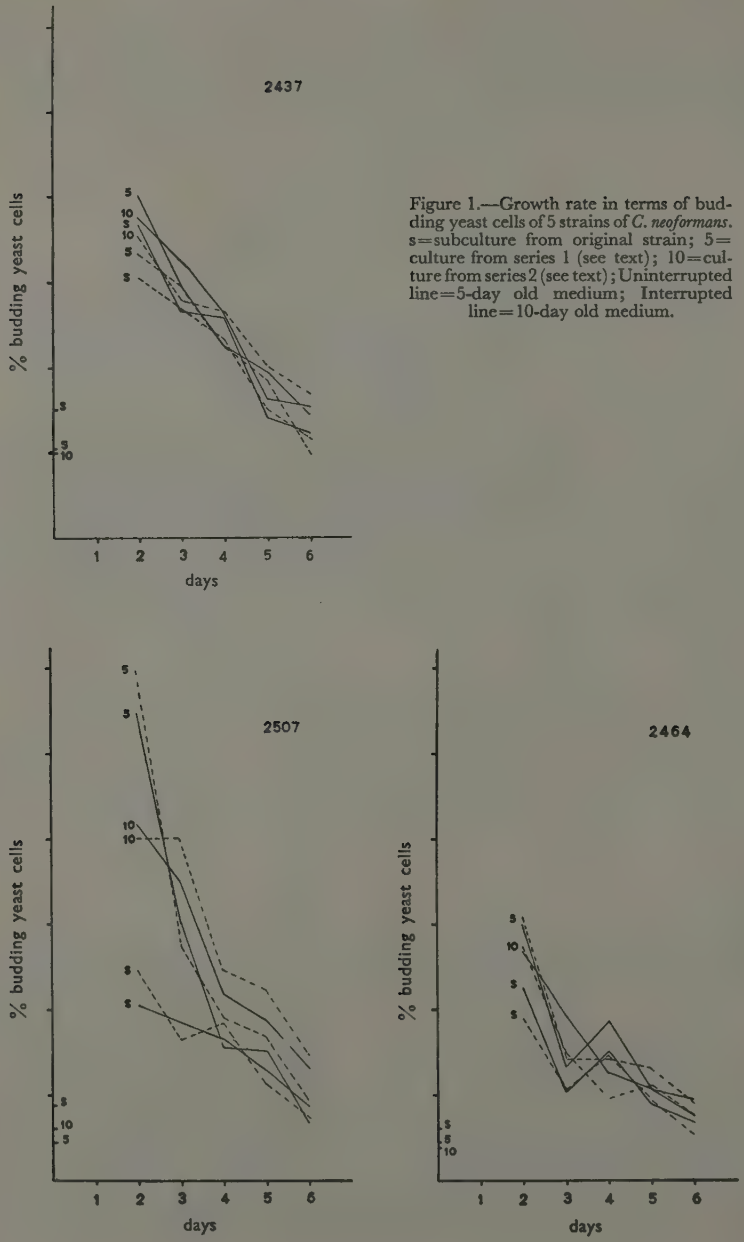

Figure 1.—Growth rate in terms of budding yeast cells of 5 strains of *C. neoformans*. s=subculture from original strain; 5=culture from series 1 (see text); 10=culture from series 2 (see text); Uninterrupted line=5-day old medium; Interrupted line=10-day old medium.

on the growth rate of the fungi, parallel experiments were performed with 5 and 15 days old medium. The plates were stored after inoculation at room temperature (approx. 20° C.) and 2 days after inoculation distinct colonies could be seen with the naked eye. Material was collected from the margin of the colonies on 5 consecutive days, suspended in physiological saline, and the number (expressed as %age) of budding cells in 1,000 cells was determined using a Buerker haemocytometer.

The tests for virulence were carried out on adult albino male mice (wt.22–24g.) belonging to a pure but not inbred strain supplied many years ago by Professor Tage Kemp, Copenhagen, and bred further in this laboratory. An intracerebral injection of 0·02ml. physiological saline suspension of *C. neoformans* was made according to the technique described by Littman and Zimmerman (1956).

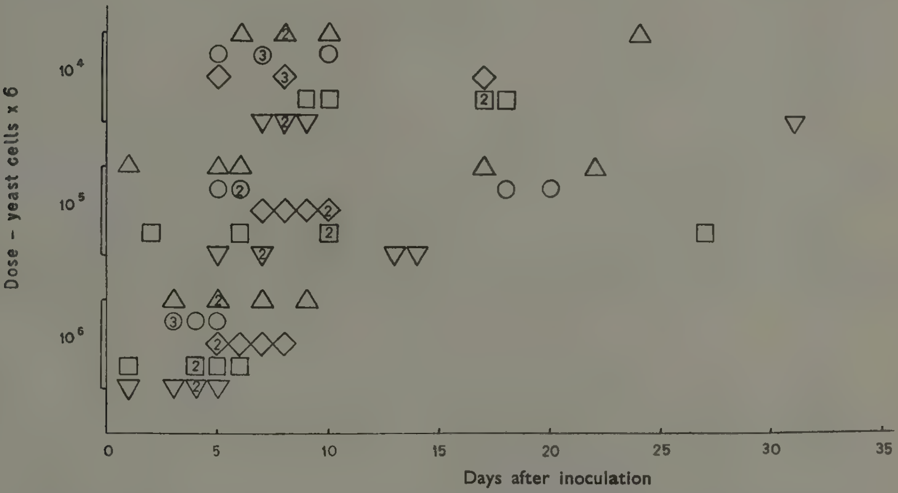

Figure 2.—Mortality of mice on intracerebral injection of serial 10-fold diluions of 5 different strains of *C. neoformans*. Groups of 5 mice. Strains : — "Malmo"; — 2507; — 2464; — 2224; — 2437.

The virulence of the 5 different strains of *C. neoformans* was compared after they had been passed through mice with intervening culture on artificial medium (series 2 in table 1). The suspension injected contained approx. $6 \times 10^6$ yeast cells. In an attempt to prolong the survival periods, groups of 5 mice received an injection of 2 serial 10-fold dilutions of the cell suspension. Fifteen groups of 5 mice were used.

To determine change in virulence of a single strain (No. 2507) the virulence of the cultures obtained after one or more passages through mice with intervening culture on the artificial medium (series 1 & 2 in table 1) was compared with the virulence of a culture maintained continuously on artificial medium for several years. Five groups of 5 mice were injected intracerebrally with 0·02 ml. of a physiological saline suspension of yeast cells in 9 different doses (1/1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256). In all 45 groups of 5 mice were used. The 1/1 dose contained about 600,000 cells/injection. The survival periods in each group are given in table 2.

## RESULTS

Fig. 1 gives the results of the examination of the growth rate in terms of budding cells. It is clear that the growth curve for *C. neoformans* did not vary with the age (5 & 15 days) of the medium at the time of inoculation. On the second day, *i.e.,* when gross colonies appeared in the plates, the proportion of budding cells was high but decreased considerably and more or less uniformly in all cultures during the following days. The difference in the number of budding cells which is frequently evident at the second and third day, both for different cultures of the same strain, and for different strains, diminished during the fourth and sixth day. The cultures which had had alternating passages through mice and culture on artificial media usually showed the highest proportion of budding cells.

In the studies of virulence it was found that there was no difference between the various strains. All 5 strains, in the doses used, proved lethal and the survival time of the animals was inversely proportional to the dose (fig. 2). It is clear from table 2 that the various sub-cultures of strain 2507 were highly virulent for mice in the doses used, that the virulence decreased only slightly with increasing dilution and that the mortality rate showed no certain tendency to rise with the number of alternating passages through mice and artificial media.

## DISCUSSION

If the results of experimental infection with micro-organisms inoculated at different times are to be compared, the experimental conditions, *e.g.,* the size of the inoculum and the virulence of the micro-organisms must be kept constant. In the present investigation the size of the inoculum *i.e.,* the number of yeast cells, could be accurately determined by counting the number of cells in the suspension. However, it is obviously hazardous to assume that all the cells are viable. The growth curves of the total number of cells and of the viable cells in a yeast culture are largely parallel during the first phases of the growth, but during the phase of decline the number of living cells is markedly reduced in relation to the total number of cells, probably because of exhaustion of the medium. Methods for calculating the number of living cells have limitations. In this investigation the growth capacity of the culture was estimated from the proportion of yeast cells showing definite signs of budding. By the time, 2 days after inoculation of Sabouraud's dextrose agar medium, the first suspension could be prepared, the proportion of budding organisms was high. During the following days, however, the proportion of budding organisms decreased markedly in all the cultures studied. This decrease in growth rate appears to agree with the phase of decline in the growth curve for viable cell counts applicable to yeasts (Morris, 1958). Since the ratio between the number of living cells and the total number of cells in a culture varies with the time from inoculation of the medium suspensions intended for injection must be prepared from cultures of similar age. Slight variation in age of the medium, however, has no appreciable effect on the rate of reproduction.

Days after injection

| Dose | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1/1 | A | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | |
| | B | 5 | 5 | 4 | 4 | 4 | 4 | [illegible] | | | | | | | | | | | | | | | | | | | | | | | | |
| | C | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | |
| | D | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | |
| | E | 5 | 5 | 5 | 5 | 5 | 2 | 1 | | | | | | | | | | | | | | | | | | | | | | | | |
| 1/2 | A | 5 | 5 | 5 | 4 | 4 | 4 | 3 | 3 | 2 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | |
| | B | 5 | 5 | 5 | 5 | 5 | 5 | 3 | 3 | 2 | 1 | 1 | | | | | | | | | | | | | | | | | | | | |
| | C | 5 | 4 | 4 | 4 | 4 | 2 | 2 | 1 | | | | | | | | | | | | | | | | | | | | | | | |
| | D | 5 | 4 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | |
| | E | 5 | 5 | 5 | 4 | 3 | 2 | 2 | 1 | 1 | | | | | | | | | | | | | | | | | | | | | | |
| 1/4 | A | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 3 | 3 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | |
| | B | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 3 | 1 | | | | | | | | | | | | | | | | | | | | | |
| | C | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 2 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | |
| | D | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 1 | | | | | | | | | | | | | | | | | | | | | |
| | E | 5 | 5 | 5 | 5 | 4 | 3 | 3 | 2 | 2 | | | | | | | | | | | | | | | | | | | | | | |
| 1/8 | A | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | 2 | 2 | | | | | | | | | | | | | |
| | B | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 1 | 1 | | | | | | | | | | | | | | | | | | | |
| | C | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 2 | 2 | 1 | | | | | | | | | | | | | | | | | | |
| | D | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 3 | 2 | 2 | 2 | 1 | 1 | | | | | | | | | | | | | | |
| | E | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 4 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | |
| 1/16 | A | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | |
| | B | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 3 | 1 | 1 | 1 | | | | | | | | | | | | | | | | | | | | |
| | C | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 3 | 3 | 2 | 1 | 1 | | | | | | | | | | | | | | | | | |
| | D | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 3 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | |
| | E | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 2 | 2 | 1 | | | | | | | | | | | | | | | | | | | | |
| 1/32 | A | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 2 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | |
| | B | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | | | | | | | | | | | | | | | | | | | | | | |
| | C | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 2 | 1 | 1 | | | | | | | | | | | | | | | | | | | |
| | D | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 3 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | | | | | | | | | | | | | |
| | E | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 2 | 2 | 2 | 1 | 1 | | | | | | | | | | | | | | | | | | |
| 1/64 | A | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 3 | 2 | 1 | 1 | | | | | | | | | | | | | | | | | | | | |
| | B | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 1 | 1 | | | | | | | | | | | | | | | | | | |
| | C | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 2 | 2 | 2 | 2 | | | | | | | | | | | | | | | | | | | |
| | D | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | | | | | | | | | | | | | | | | |
| | E | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 2 | 2 | 2 | 2 | 1 | | | | | | | | | | | | | | | | | | |
| 1/128 | A | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 3 | 3 | 2 | 2 | 2 | 1 | | | | | | | | | | | | | | | | |
| | B | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 |
| | C | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 3 | 3 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | |
| | D | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | |
| | E | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 3 | 3 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | |
| 1/256 | A | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 3 | 1 | 1 | | | | | | | | | | | | | | | | | | |
| | B | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 3 | 3 | 3 | 2 | 2 | 1 | | | | | | | | | | | |
| | C | 5 | 5 | 4 | 4 | 4 | 4 | 4 | 3 | 3 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | |
| | D | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 3 | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | | | | |
| | E | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 3 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | | | | | | | | | | | | | | | |

Table 2.—Determination of virulence of 5 cultures of *C. neoformans* strain 2507. A=subculture from original strain; B=culture on artificial medium after one mouse passage (5 days); C=culture on artificial medium after one mouse passage (10 days); D=culture from series 1 (see table 1) 57 mouse-medium passages; E=culture from series 2 (see table 1) 41 mouse-medium passages. Values indicate number of living mice in groups of 5 mice that had received an intracerebral injection. Uninterrupted line indicates that no mice, or at most 1 mouse, was still alive.

Benham (1955) and Osswald & Seeliger (1960) found that *Cryptococcus* strains isolated from various sources differed considerably in their pathogenicity for animals. All 5 strains of *C. neoformans* studied in the present investigation showed high pathogenicity for mice and no difference in this respect could be demonstrated. Neither was any increase in virulence detected in the strain which had been passed through mice with intervening culture on artificial media.

The influence of several successive mouse passages without intervening culture on artificial medium on the virulence of *C. neoformans* is being investigated.

## Zusammenfassung

Es wurden 5 Stämme von Cryptococcus neoformans in Hinsicht auf ihre Vermehrung auf festem Substrat und Virulenz an Mäu studiert. Die Vermehrung—ausgedrückt in der Anzahl 'sprossender Organismen'—begann innerhalb 48 Stunden nach der Inokulation zu sinken. Es wurde kein Unterschied der Kulturen gefunden, die auf verschiedenen alten Substraten (5 und 15 Tage alt) gewachsen sind.

Es wurde weiterhin kein Unterschied der Virulenz bei den verschiedenen Stämmen an der Maus gefunden. 42–57 Mäuse-Passagen mit 5–10 Tage dazwischen-liegender Kultur von festem Substrat konnten keine Steigerung der Virulenz der Pilze nach intracerebraler Injektion hervorrufen.

Die beschriebenen Experimente sind ein Glied einer grösseren experimentellen Studie über die Pathogenese des *Cryptococcus neoformans* bei Mäusen, die später publiziert wird.

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# *NANNIZZIA INCURVATA* GEN. NOV., SP. NOV., A PERFECT STATE OF *MICROSPORUM GYPSEUM* (BODIN) GUIART ET GRIGORAKIS

PHYLLIS M. STOCKDALE

*Commonwealth Mycological Institute, Kew.*

Two strains of *Microsporum gypseum* (Bodin) Guiart et Grigorakis isolated from human skin lesions have been found to produce cleistothecia when grown on hair or other keratinaceous material on the surface of soil. The cleistothecia originate as club-shaped antheridia surrounded by coiled ascogonia. They are white at first, becoming light buff and finally yellowish buff as the asci become mature. The asci are globose to ovate and contain 8 yellow, lenticular, smooth-walled ascospores, 2·8—3·5×1·5—2·0μ diam. The peridial hyphae are branched in a verticillate manner and are composed of thick-walled, asperulate, hyaline cells with one or more symmetrical constrictions. There are numerous free ends and appendages of 3 kinds—long, straight or loosely coiled hyphae, spiral hyphae and macroconidia similar to those formed on the vegetative mycelium.

The fungus has characters which distinguish it from all known genera of the Gymnoascaceae and the name *Nannizzia incurvata* gen. nov., sp. nov. is proposed.

The first suggestion that the dermatophytes are related to the Gymnoascaceae was made by Matruchot & Dassonville (1899a, b) who found that certain species of the Gymnoascaceae produced asexual spores similar to those of the dermatophytes. They also compared the spirals of *Trichophyton* with those found on the peridial hyphae of *Ctenomyces serratus*. They came to the conclusion that the dermatophytes were the imperfect forms of Gymnoascaceae, possibly of *Ctenomyces* or possibly of a related genus as yet unknown. Later (1900) they found that isolates of a species of *Trichophyton* produced clumps of hyphae which were similar to cleistothecia but contained only conidia, and which they termed 'fruits conidiens'. These bodies, which had been found previously by Eidam (1880) in *C. serratus*, have since been described by many other workers including Langeron & Milochevitch (1930) who obtained them in *T. lacticolor*, *T. interdigitale*, *T. granulosum*, and *T. radiolatum*. On this and other evidence they transferred these species, now considered to be conspecific with *T. mentagrophytes*, to *Ctenomyces*.

There are few records of the existence of a perfect state in the dermatophytes and most of these have been discredited. Nannizzi (1927) described cleistothecia in a strain of *Sabouraudites* (*Microsporum*) *gypseum* isolated from a skin lesion (fig. 1). He obtained the cleistothecia by growing the isolate for several months on forest soil mixed with feathers and old skin. They consisted of a central mass of asci 7–8 μ diam., each containing 8 oval, smooth, hyaline ascospores, 4·5×3 μ diam. The asci were surrounded by a wall constituted of a complex of abundantly ramified, hyaline, smooth-walled hyphae, 4–5 μ diam., from which arose, towards the outside of the cleistothecium, thicker hyphae, 6–8 μ diam., arcuate, simple or dichotomously branched, sometimes unilaterally ramified, composed of cells with thin, echinulate walls and granular contents, short and restricted in the middle. Nannizzi considered his isolate to be a species of *Gymnoascus* and proposed the name *G. gypseus*.

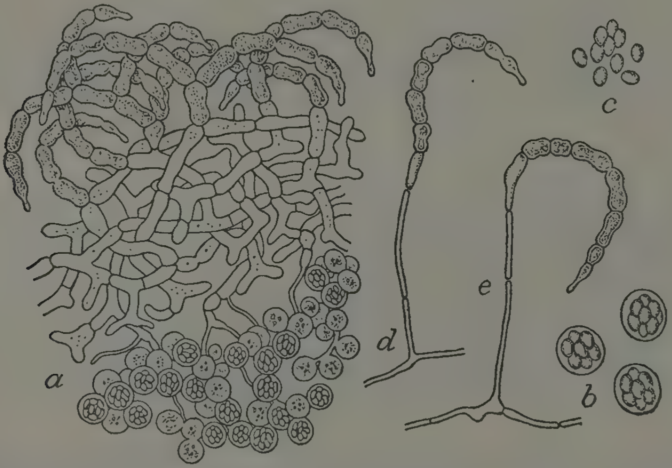

**Figure 1.**—*Gymnoascus gypseus* (from Nannizzi, 1927). *a*, portion of the peridium; *b*, asci; *c*, ascospores.

## OBSERVATIONS

The present study concerns chiefly a strain of *M. gypseum* (D 132) which was isolated by Whittle (1954) from a skin lesion on a worker in a plant nursery. In March, 1959, cultures of this strain which had been recovered from freeze-dried material prepared soon after isolation produced a few small yellow-buff clumps of hyphae which on microscopic examination were found to be mature cleistothecia containing asci. These cultures were on 4% malt agar, but cleistothecia were no longer formed on further subculture on to this medium or on to a wide variety of other mycological and bacteriological media.

Dawson & Gentles (1959) found that *Keratinomyces ajelloi* formed cleistothecia on child's hair on the surface of soil, and this method was therefore tried, and found to be successful, for *M. gypseum.* Petri dishes were half-filled with soil and short lengths of child's hair were placed on the surface. The plates were autoclaved at 20 lb./20 min., inoculated with an aqueous spore suspension and kept at room temperature. After 4–6 days the hairs became overgrown by a pale buff, fluffy mycelium in which the cleistothecia were first visible after 8–10 days as small, white clumps of hyphae. These gradually enlarged and became pale buff and finally yellowish buff, usually 350–500 μ diam. but occasionally reaching 650 μ diam. excluding the appendages.

Microscopic examination of the mycelium at 5-8 days shows numerous cleistothecial initials consisting of a central club-shaped, septate antheridium surrounded by a coiled ascogonium (fig 2a). These gametangia always arise from separate hyphae. After fusion has taken place the ascogonium becomes septate and produces croziers (fig. 2d). At the same time the initial becomes surrounded by a thick weft of hyphae which grow from the parent hypha and basal cells of the antheridium (fig. 2b). Apparently these hyphae give rise to the peridial hyphae. At first they are thin-walled, smooth, occasionally septate with branches growing immediately below the septa (fig. 2e). Later the hyphal walls become moderately thickened and densely asperulate, further septa are formed and the hyphae towards the outside of the cleistothecium swell unequally so that at maturity (fig. 2g) they consist of cells with up to 3 more or less symmetrical constrictions along their length, usually up to 7–8 μ diam. but occasionally reaching 11 μ in their thickest parts. Meanwhile further branches are formed in succession at the same level and the original hypha is pushed to one side of the main axis. As many as 5 branches at varying stages of development have been seen to emerge in verticillate formation from the tip of 1 cell. In this way the initial becomes surrounded by a thick mesh of closely interwoven, branching hyphae (pl. 1a). The branches are almost always curved in towards their main axis but occasionally a branch which curves over the cleistothecium is seen (fig. 2h).

The peridial hyphae usually end as blunt projections, but many extend as smooth-walled, septate, occasionally branching hyphae which are either straight to loosely coiled or tightly coiled in spirals with up to 30 twists. The straight to loosely coiled hyphae (pl. 1c) extend up to 300 μ from the peridium and are 3·0–4·5 μ tapering to 1·5–2·0 μ diam. The tightly coiled hyphae appear to be generally much longer than this and are usually not more than 3·0–3·5 μ diam. at the base. Thick-walled, asperulate, spindle-shaped macroconidia with up to 5 cross-walls, 40–57 μ × 10–12·5 μ, identical with those formed on the vegetative mycelium, are sometimes formed in large numbers on the peridial hyphae (pl. 1b). They become detached very easily and usually only the blunt ends of the hyphae which had borne them are seen.

When the cleistothecium is crushed the peridial hyphae collapse and stick together and the asci are released. These are globose to ovate, usually 5–6 μ diam. but occasionally up to 7 μ diam., and contain 8 ascospores. The ascospores are yellow in mass, smooth-walled, lenticular, with no equatorial ridge or furrow, 2·8–3·5 μ × 1·5–2·0 μ (fig. 2f, pl. 1d).

Single ascospore cultures have revealed that the strain is heterothallic. Some single spore isolates, when grown alone on soil and hair, form abortive cleistothecia consisting of a mass of peridial hyphae but no asci. These bodies appear to arise from organs similar to antheridia without ascogonial coils (fig. 2c).

Recently, another strain of *M. gypseum* (D 134) which forms cleistothecia identical with the ones described above was isolated at the London School of Hygiene and Tropical Medicine from skin lesions. None of 12 other strains of *M. gypseum* formed cleistothecia when grown alone on soil and hair but in experimental crosses with single ascospore isolates of D 132 (SA2+ and SA3−) 4 formed cleistothecia in combination with SA2+ and 3 with SA3−. None of the remaining strains have been induced to form cleistothecia in combination with

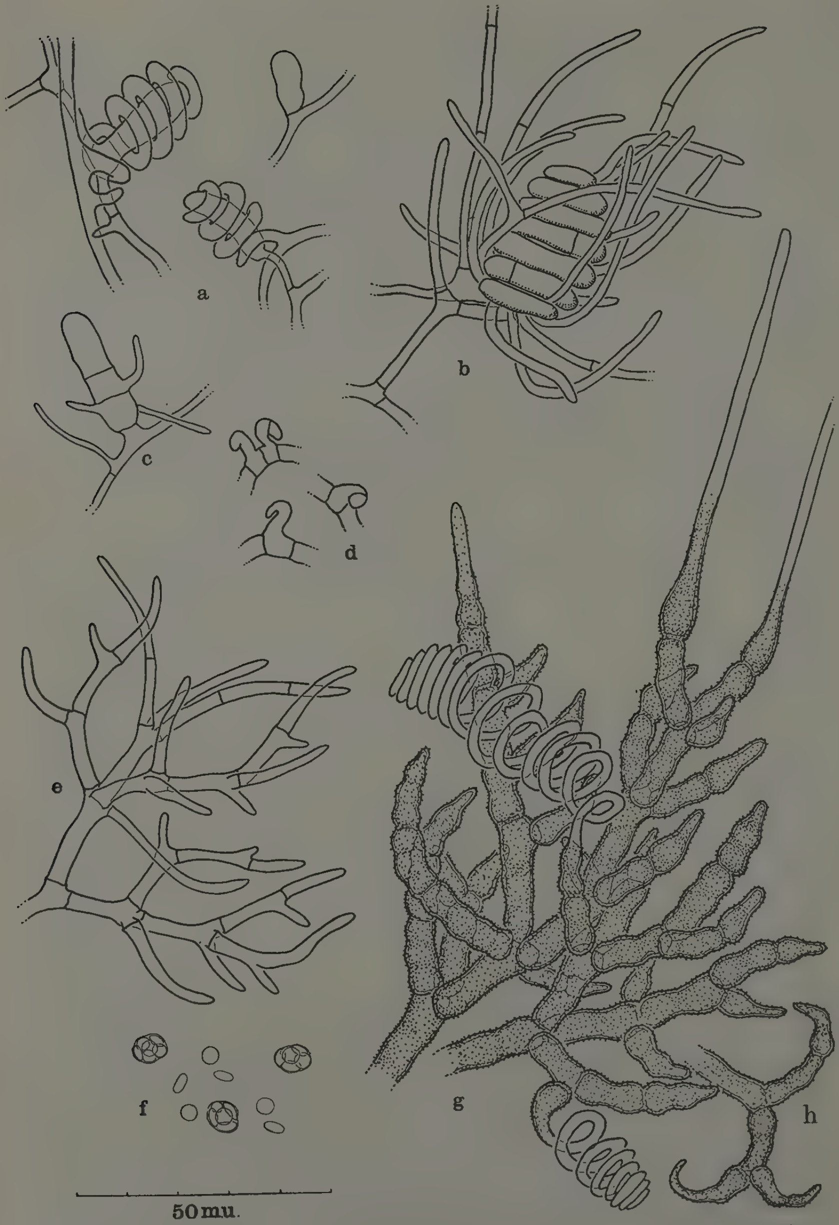

Figure 2.—*Nannizzia incurvata*. *a*, club-shaped antheridia surrounded by coiled ascogonia; *b*, a cleistothecial initial becoming surrounded by hyphae growing from the antheridium; *c*, hyphae growing from an antheridium in the absence of an ascogonial coil; *d*, croziers developing on an ascogonium; *e*, young peridial hyphae; *f*, asci and ascospores; *g-h*, mature peridial hyphae.

either of these single spore isolates or with each other. This may be partly explained by the fact that cleistothecia were formed very sparingly in crosses with SA3— and may have been missed in some instances. Details of the strains examined are given at the end of the paper.

Child's hair and chicken feathers were found to be the best source of keratin but a variety of other sources including adult human hair, skin, and nails, sheep's wool, swan's feathers, snake's skin, and hair from cats, dogs, horses, cattle, and guinea-pigs also supported cleistothecium formation to some extent, as did malt agar (2 % or 4 %) and rabbit dung agar. Other commonly used media such as glucose-peptone, 3 % peptone, and potato-dextrose agars and sterile barley grains were not suitable.

## Discussion

In the present system of classification of the Gymnoascaceae as presented by Benjamin (1956) and Kuehn (1958) this fungus agrees most closely with the genus *Arthroderma*. Benjamin points out that the type species of this genus, *A. curreyi* Berkeley, has been described frequently in the literature as *Ctenomyces serratus*. Eidam (1880) described two stages of this species—a resting stage with comb-like appendages, now recognised as the cleistothecium of *C. serratus* (Benjamin, 1956), and an ascigerous stage which had been described previously by Currey (1854). Currey had suggested the name *Arthroderma* because of the jointed nature of the hyphae and Berkeley (1860) took up this name and called Currey's fungus *A. curreyi*. It was with fungi resembling *A. curreyi* that early authors compared dermatophytes when they considered the latter to be related to *Ctenomyces*.

No type specimen of *A. curreyi* has been found but collections under the name *Illosporium curreyi* (Berk.) Sacc. in the herbarium of the British Museum (Nat. Hist.) have been examined. The cleistothecium of *A. curreyi* resembles that described here in many ways. In both the peridium consists of a branching network of hyaline, asperulate, septate hyphae; but in *A. curreyi* the branching is consistently dichotomous and never verticillate. Also, in *A. curreyi*, the outer hyphae, which curve over the cleistothecium and not away from it, are composed of cells with small projections on the outside of the curve (fig. 3). When viewed from above these cells appear symmetrical in outline. Long, straight or loosely coiled extensions of the peridial hyphae have never been seen in *A. curreyi* and in this species the spiral appendages are frequently borne laterally. These differences are considered to be of generic significance and it is proposed to establish a new genus for the perfect state described here.

### *Nannizzia* gen. nov.

*Cleistothecia* globosa. *Peridium* ex reticulo hypharum hyalinarum, septatarum, verticillatim ramosarum compositum. Cellae modice crasso-parietales, dense asperulatae, plus vel minus aequaliter constrictae. Hyphae apicibus liberis numerosis, appendiculis triformibus: (1) Hyphae elongatae, graciles, parietibus laevibus, septatae, aliquando ramosae, rectae vel haud strictim volutae. (2) Hyphae elongatae, graciles, parietibus laevibus, septatae, aliquando ramosae,

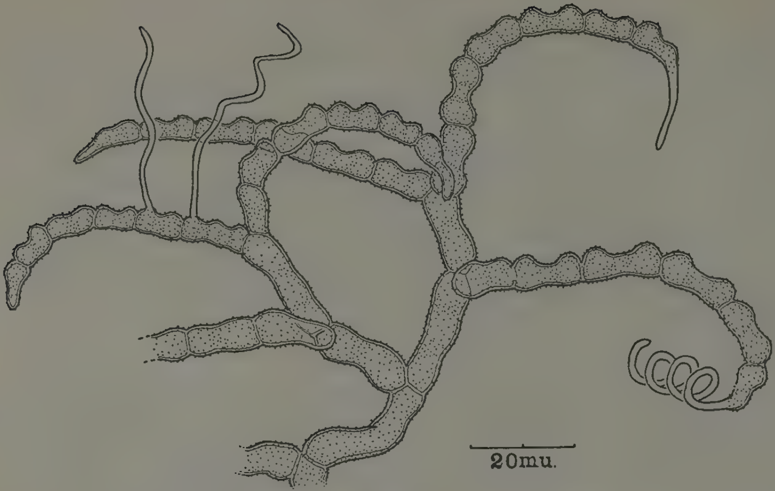

Figure 3.—*Arthroderma curreyi*. Part of a mature peridial hypha.

strictim volutae. (3) Macroconidia. *Asci* globosi vel ovati, hyalini, octospori. *Ascospori* lutei, Lenticulares. *Typus: Nannizzia incurvata*

*Cleistothecia* globose. *Peridium* consisting of a network of hyaline, septate, verticillately branched hyphae. Cells moderately thick-walled, densely asperulate, more or less symmetrically constricted. Hyphae with numerous free ends, and with appendages of three kinds: (1) Elongate, slender, smooth-walled, septate, occasionally branching, straight or loosely coiled hyphae. (2) Elongate, slender, smooth-walled, septate, occasionally branching, tightly coiled hyphae. (3) Macroconidia. *Asci* globose to ovate, hyaline, 8-spored. *Ascospores* yellow, lenticular. Named after Dr. Arturo Nannizzi.
*Type: Nannizzia incurvata*

**Nannizzia incurvata** sp. nov.
*Cleistothecia* globosa, pallido-bubalina vel flavo-bubalina, 350–650 μ diam. appendiculis exclusis. *Hyphae peridii* pallido-bubalinae, hyalinae, septatae, verticillatim ramosae, usque ad 5 ramos ad axem principem plerumque curvatos gerentes. Cellae modice crasso-parietales, dense asperulatae, 1–3 plus vel minus aequaliter constrictionibus plerumque usque ad 7–8 μ diam. aliquando 11 μ diam. Hyphae apicibus liberis numerosis, appendiculis triformibus: (1) Hyphae elongatae, graciles, parietibus laevibus, septatae, aliquando ramosae, usque ad 300 μ longae, ab 3·0–4·5 μ ad 1·5–2·0 μ diam. sensim diminuentes, rectae vel haud strictim volutae. (2) Hyphae elongatae, graciles, parietibus laevibus, septatae, aliquando ramosae, ab 2·5–3·5 μ ad 1·5–2·0 μ diam. sensim diminuentes, usque ad triciens strictim volutae. (3) Macroconidia parietibus crassis, asperulata, fusiformia, 40–57 μ × 10–12·5 μ, 1–5 septis. *Asci* globosi vel ovati, 5–7 μ diam., octospori. *Ascospori* lutei, parietibus laevibus, lenticulares, 2·8–3·5 μ × 1·5–2·0 μ.

*Stat. conid. Microsporum gypseum* (Bodin) Guiart et Grigorakis.
*Typus:* Herb. I.M.I. 82777.

*Cleistothecia* globose, pale-buff to yellow-buff, 350–650 μ diam. excluding appendages. *Peridial hyphae* pale buff, hyaline, septate, verticillately branched with up to 5 branches which usually curve in towards their main axis. Cells moderately thick-walled, densely asperulate, with 1–3 more or less symmetrical constrictions, usually up to 7–8 μ diam. but occasionally reaching 11 μ diam. Free ends numerous and appendages of three kinds: (1) Elongate, slender, smooth-walled, septate, occasionally branched hyphae, up to 300 μ long, 3·0–4·5 μ diam. at the base tapering to 1·5–2·0 μ diam., straight or loosely coiled. (2) Elongate, slender, smooth-walled, septate, occasionally branched hyphae, 2·5–3·5 μ diam. at the base tapering to 1·5–2·0 μ diam., tightly coiled with up to 30 spirals. (3) Macroconidia thick-walled, asperulate, spindle-shaped, 40–57 μ × 10–12·5 μ, with 1–5 septa. *Asci* globose to ovate, 5–7 μ diam., 8-spored. *Ascospores* yellow, smooth-walled, lenticular, 2·8–3·5 μ × 1·5–2·0 μ.
*Stat. conid.: Microsporum gypseum* (Bodin) Guiart et Grigorakis.
*Habitat:* In culture, 8 years after isolation from human skin lesions.
*Type:* Herb. I.M.I. 82777.

The cleistothecia described by Nannizzi for *M. gypseum* resemble those of *Nannizzia incurvata* in the shape of the cells of the peridial hyphae, but differ from them in having peridial hyphae which appear to branch dichotomously and curve over the cleistothecium as in *Arthroderma,* and which do not carry any form of appendage. Also the ascospore measurements reported by Nannizzi are larger than those for *N. incurvata.* This suggests that *M. gypseum* may be the imperfect state of more than one species. This is supported by the observation that 5 of 12 strains identified as *M. gypseum* failed to form cleistothecia alone or in combination with single ascospore isolates of *N. incurvata.*

## STRAINS EXAMINED

*Nannizzia incurvata* :

| | |
|---|---|
| I.M.I. 82777 | Culture isolated from skin lesions on a worker in a carnation nursery, Hertfordshire, 1951. C. H. Whittle—L.S.H.T.M. (D 132). (As *M. gypseum.*). SA2+ and SA3− (single ascospore isolates). |
| I.M.I. 82776 | Culture isolated from skin lesions, Reading, 1960. L.S.H.T.M. (D 134). (As *M. gypseum*). |

Cultures identified as *Microsporum gypseum* which formed cleistothecia in combination with I.M.I. 82777–SA2+ :

| | |
|---|---|
| D 117 | Isolated from human skin lesions, London, 1948. |
| D 123 | Isolated from human hair infection, 1949. |
| D 126 | Isolated from human skin lesions, Ireland, 1951. |
| D 127 | Isolated from human skin lesions, London, 1950. |

Cultures identified as *Microsporum gypseum* which formed cleistothecia in combination with I.M.I. 82777–SA3− :

| | |
|---|---|
| D 129 | Isolated from soil, U.S.A., 1951. M. A. Gordon (A 584). |
| D 130 | Isolated from human skin lesions, Belfast, N. Ireland, 1952. |
| D 135 | Isolated from human skin lesions, Kent, 1960. |

Cultures identified as *Microsporum gypseum* which have failed to form cleistothecia in combination with single spore isolates of I.M.I. 82777 or with each other :

| | |
|---|---|
| I.M.I. 82775 | Isolated from rose garden soil, Bath, Somerset, 1956. P. Stockdale. |
| I.M.I. 82774 | Isolated from farmyard soil, Box, Wiltshire, 1957. P. Stockdale. |
| D 116 | Isolated from human skin lesions, New Zealand, 1947. M. Marples. |
| D 128 | Isolated from soil, U.S.A., 1951. M. A. Gordon (A 582). |
| D 131 | Isolated from human hair infection, 1952. |

*Arthroderma curreyi* :

On feathers, Hereford, 1872. Mr. Renney. Herb. B.M. (Nat. Hist.). (As *Illosporium curreyi*).

On twigs and leaves (indet.), nr. Selby, Yorkshire, 1918. Annie Lorraine Smith. Herb. B.M. (Nat. Hist.). (As *Illosporium curreyi*).

Cultures with numbers prefixed 'D' are from the Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine.

## Zusammenfassung

Zwei von menschlichen Hautläsionen isolierten und auf der mit Haar oder anderem Keratin Material gemischten Erde gezüchteten *Microsporum gypseum* (Bodin) Guiart et Grigorakis Stämme entwickelten Cleistothezien. Diese entstehen aus von spiral-förmigen Ascogonien eingeschlossenen keulen-förmigen Antheridien.Zuerst weiss, sie werden beige, und endlich gelb-beige wenn die Schläuche reifen. Diese Schläuche sind globos und enthalten 8 gelbe, linsenförmige, glattwändige Ascosporen ($2{,}8-3{,}5\mu \times 1{,}5-2{,}0\mu$). Die peridiale Hyphen sind vertizillat gezweigt und bestehen aus dickwändigen, rauhen Zellen mit einer oder mehreren Verengungen. Es gibt viele losen Ende und Anhänge von drei Formen : Makrokonidien, welche den sich auf dem vegetativen Myzel entwickelnden ähnlich sind; Spiralhyphen; und lange, gerade oder leicht gewickelte Hyphen.

Der Pilz besitzt Eigenschaften die ihn von allen bekannten Gattungen der Gymnoaskazeën entscheiden. Der Name *Nannizzia incurvata* gen. nov., sp. nov., wird vorgeschlagen.

## Acknowledgements

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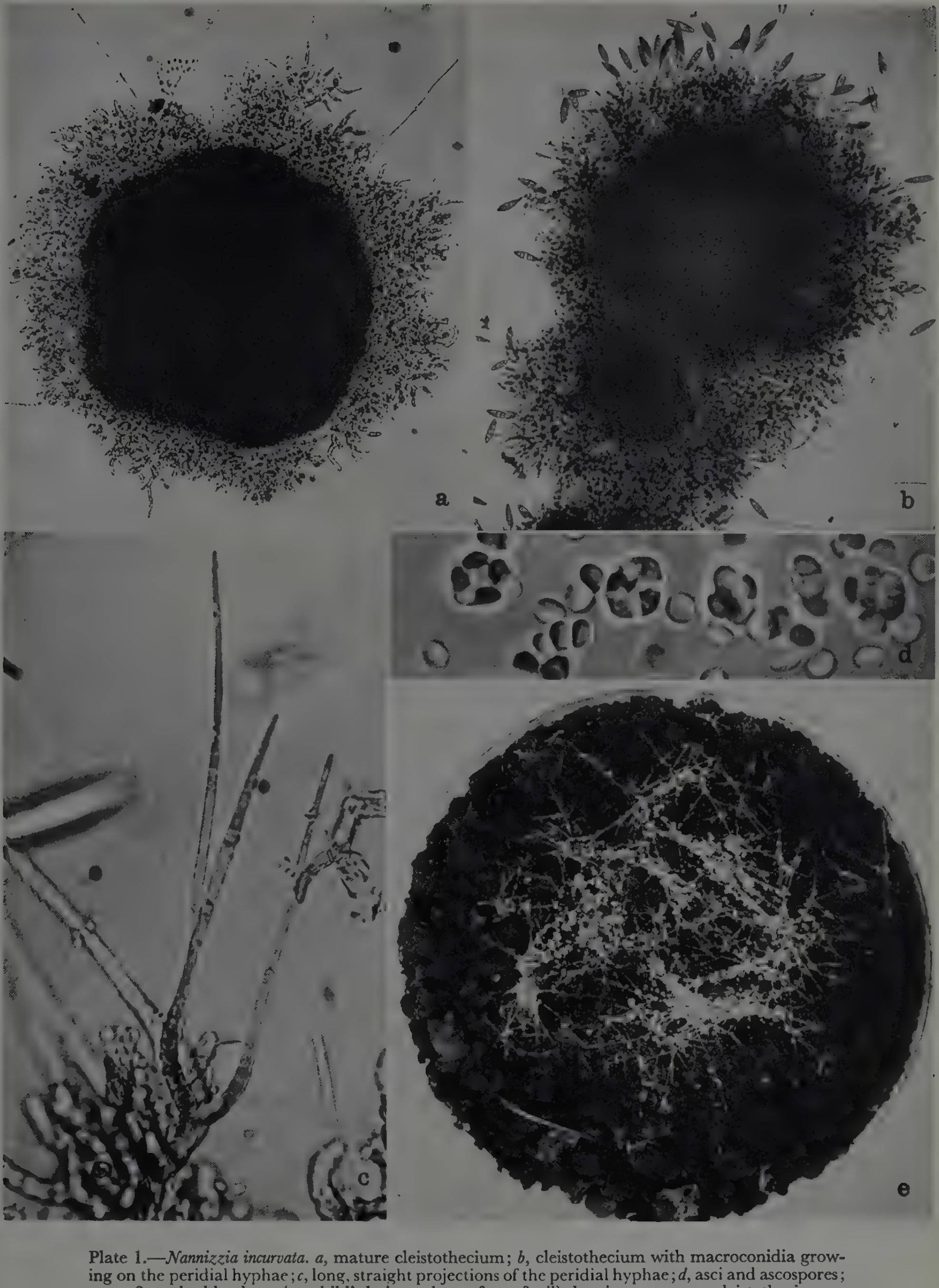

Plate 1.—*Nannizzia incurvata*. *a*, mature cleistothecium; *b*, cleistothecium with macroconidia growing on the peridial hyphae; *c*, long, straight projections of the peridial hyphae; *d*, asci and ascospores; *e*, 3-week-old culture (on child's hair on the surface of soil) showing numerous cleistothecia.

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# THE PERFECT STATES OF *KERATINOMYCES AJELLOI* VANBREUSEGHEM, *TRICHOPHYTON TERRESTRE* DURIE & FREY AND *MICROSPORUM NANUM* FUENTES.

Christine O. Dawson and J. C. Gentles

*Department of Bacteriology, University of Glasgow.*

During investigations of soil samples from various districts in the West of Scotland using Vanbreuseghem's (1952) hair-bait method, *Keratinomyces ajelloi, Trichophyton terrestre, Microsporum gypseum* and *Arthroderma curreyi* were isolated. These fungi, and *Microsporum nanum* isolated from ringworm-infected pigs in Kenya, have been studied in culture on unsterilised soil. Descriptions are given of the perfect stages of *K. ajelloi* and *M. nanum* which were obtained in soil culture and that of *T. terrestre* which arose spontaneously in an agar slope culture. It is proposed that the perfect forms of *K. ajelloi* and *T. terrestre* be included in the genus *Arthroderma* Currey as *A. uncinatum* sp. nov. and *A. quadrifidum* sp. nov. respectively, and that the perfect form of *M. nanum* be put in the genus *Nannizzia* Stockdale as *N. obtusa* sp. nov.

Certain morphological characters of the ringworm fungi have long been accepted as an indication of a close relationship with the Gymnoascaceae. Despite many careful studies however, conclusive proof of this relationship, the discovery of the perfect stage of a dermatophyte, has eluded mycologists for many years. The report by Nannizzi (1927) on the perfect stage of *Microsporum gypseum* was, for various reasons, not generally accepted.

In an effort to induce sexual reproduction Vanbreuseghem & Van Brussel (1952) studied dermatophytes in culture on soil and on various media supplemented with soil extracts. They were unsuccessful in their search for perfect stages but found that soil was clearly a more natural substrate than agar media. Using the 'hair bait' technique by which Vanbreuseghem (1952) isolated *Keratinomyces ajelloi* we reisolated it from soil and discovered its perfect stage (Dawson & Gentles, 1959). *K. ajelloi* has been reported by Georg, Kaplan, Ajello, Williamson & Tilden (1959) as the cause of ringworm in a squirrel and may therefore be considered a dermatophyte. In 1959 Stockdale (personal communication) identified the perfect stage of *M. gypseum* which has subsequently been reported by Griffin (1960) from Australia and by Szathmary & Herpay (1960) from Hungary.

Recently, during investigations in which soil culture was used extensively, we have obtained the perfect stages of the dermatophyte *M. nanum* and of the non-pathogenic fungus, *Trichophyton terrestre*. In this paper the cleistothecia of these 2 fungi, *K. ajelloi* and *Arthroderma curreyi* are described and methods of soil culture are given.

## Materials and Methods

Samples of surface soil collected from a number of districts in the West of Scotland were placed in Petri dishes and baited with autoclaved horse mane or tail hair. At first the dishes were incubated at 28° C. but later it was discovered that 24° C. gave better results and the latter temperature was used. The Petri dishes were examined at intervals of 2–3 days and fungi colonising the hair bait were tentatively identified by microscopic examination. The identification was confirmed by culture of small fragments of colonized hair on nutrient agar supplemented with penicillin (20 units/ml.), streptomycin (40 units/ml.), and actidione (0·5 g./l.). The soil plates were moistened with sterile water when necessary and retained for at least 8–10 weeks. They were examined carefully at intervals for evidence of sexual reproduction and single ascospore isolations were made with the Singer micromanipulator when cleistothecia were formed.

Dermatophytes, and those keratinophilic fungi which showed no evidence of sexual reproduction when originally isolated, were studied by culture on the soils which had been found negative for keratinophilic fungi. The fungi were grown singly, in combination with other isolates of the same species, and in combination with single ascospore strains when these were available. Inoculation was carried out by pipetting spore-mycelial suspensions, prepared in tissue grinders, over the autoclaved bait on the unsterilised soil surface. This is the procedure referred to in all cases when it is stated that isolates were tested for ability to reproduce sexually and when single ascospore strains were used to ascertain whether a fungus was homothallic or heterothallic. Media of various types, usually 4% malt extract agar, were used for conservation of pure cultures of all isolates and of monospore strains. Studies of sexual reproduction were also made on complete medium (Pontecorvo, Roper, Hemmons, MacDonald & Bufton, 1953), Czapek-dox agar containing 1% peptone and on other common media.

## Results

### **Keratinomyces ajelloi** Vanbreuseghem

*K. ajelloi* was isolated on 17 occasions from soil in the West of Scotland. Two strains (A.971 and B.139) received from Dr. Ajello, U.S.A., and 2 obtained from Dr. Rieth, Germany were also studied. Strain B.139 is that isolated by Georg *et al.* (1959) from ringworm lesions on a squirrel. One of the cultures received from Rieth formed microconidia only and was morphologically quite different from typical *K. ajelloi* strains.

The perfect stage was formed by *K. ajelloi* on the horse hair bait at the time of isolation from 7 soil samples collected from 3 different districts. Soil culture of single ascospore strains showed that the fungus was heterothallic (Dawson & Gentles, 1959) and these 7 soils must therefore have contained both mating types. *K. ajelloi* from the remaining 10 soil samples reproduced asexually only. The isolates from these soils and those obtained from Ajello and Rieth were grown on unsterilised soil with plus and with minus single ascospore strains. Two of them (Ajello A.971., Rieth, normal strain) were thus induced to form cleistothecia although they did not reproduce sexually when grown alone on baited soil. None of the other isolates could be induced to reproduce sexually, and with one exception all were of the unpigmented variety reported first by Ajello (1953). All natural isolates capable of sexual reproduction and all single ascospore strains formed the diffusible vinaceous-red pigment on glucose-peptone agar reported by Vanbreuseghem (1952) in his original description. As a general rule therefore it may be said that pigment production by *K. ajelloi* is indicative of a potential ability to reproduce sexually. The single pigment-forming strain which could not be induced to form cleistothecia was Rieth's atypical microconidial variant.

Examination of early stages in cleistothecial development show the initials to be composed of a club-shaped antheridium enclosed within a spiralled ascogonium (pl. 1b). Further development is difficult to follow because of the rapid formation of the peridium (pl. 1c) but by crushing preparations of later stages ascogenous branches with developing asci can easily be seen (pl. 1e).

**Trichophyton terrestre** Durie & Frey

Since its isolation from soil in Australia by Durie & Frey (1957) *T. terrestre* has been found in soil in England (Stockdale, 1958), in California (Plunkett & Orr, personal communication, 1959) and in Germany (Rieth, personal communication, 1960). Four isolates were obtained from soil in the West of Scotland. The fungus has also been cultured on numerous occasions from the hair of clinically normal wild rats and twice from small papular lesions on a horse. A culture of *T. terrestre* from soil in California was obtained from Professor O. A. Plunkett and Mr. G. F. Orr and 3 cultures, 2 of which had been isolated by Miss P. M. Stockdale and the other by Dr. E. Beatrix Durie and Dr. Dorothea Frey, were obtained for study from the London School of Hygiene and Tropical Medicine.

None of the *T. terrestre* isolates showed evidence of sexual reproduction when first recovered from soil and many attempts to induce sexual reproduction by culture on unsterilised soil were unsuccessful. The perfect stage first developed in culture on a slope of Czapek-dox agar medium supplemented with 1% peptone. The isolate, which came from soil in the West of Scotland, had been conserved in the laboratory and routinely sub-cultured on various agar media for almost a year. Single ascospore studies showed *T. terrestre* to be heterothallic and also that temperatures above 25° C adversely affected sexual reproduction. It is now clear that incubation at 28° C. was the reason for the failure to obtain sexual reproduction in soil culture. Recent attempts to induce sexual reproduction by culturing isolates from different sources together in soil were successful at 24° C. and unsuccessful at 28° C.

Cleistothecia were produced when single ascospore strains were mated with natural isolates and it was found that for the majority of isolates it was of no consequence whether the monospore strains were of the plus or minus type. Such isolates were therefore potentially capable of sexual reproduction when cultured alone although attempts to induce them to do so were unsuccessful. They required the stimulation of mating with another isolate or a monospore strain. The numbers of cleistothecia obtained by mating single ascospore strains with wild isolates showed that the latter were usually predominantly of one mating type and single conidial isolates made from a number of the natural mixed strains were found to be of the mating type which had predominated in the original isolate.

Three isolates were found by mating studies to be single mating types. The sources of these were soil (Stockdale), the hair of a wild rat, and a papular lesion on a horse. We have been unable to induce sexual reproduction in the isolates of Durie and Frey or Plunkett and Orr. These, in soil culture, produced cleistothecial-like structures with a peridium typical of a *T. terrestre* cleistothecium but enclosing a mass of microconidia. These conidial structures, similar to those described by Eidam (1880) for *Ctenomyces serratus* have also been found in soil cultures of sexual strains after incubation at 28° C. for 5–6 weeks.

The perfect stage of *T. terrestre* has also been obtained by Dr. Rieth and his colleagues of the Universitäts-Hutklinik, Hamburg, Germany (personal communication, 1960).

### **Arthroderma curreyi** Berkeley

*A. curreyi* was first described by Currey (1854) who in his very brief report suggested the name *Arthroderma* to describe the jointed nature of the peridium. Berkeley (1860) validated the name *Arthroderma curreyi*. According to Benjamin (1956) *A. curreyi* has been reported in the literature many times under the name of *Ctenomyces serratus*.

Only one isolate of *A. curreyi* was obtained from soil in Scotland and a second strain for study was obtained from Dr. Kuehn, Iowa, U.S.A. Single ascospore strains of both isolates formed cleistothecia freely in soil culture and on complete agar medium (Pontecorvo *et al.*, 1953). The fungus is therefore homothallic.

Probably as a result of the hitherto unique character of the cells of the peridium the form of branching of the peridial hyphae of *A. curreyi* has not been reported in detail. Benjamin (1956) and Kuehn (1958) describe the peridium as composed of 'hyaline hyphae, branched and anastomosed, thick-walled, densely asperulate, with constricted, symmetrical or asymmetrical, dumb-bell shaped cells: thin walled, septate, elongate spiral appendages occurring terminally or laterally'. This description covers the characters exhibited by the cleistothecia of *K. ajelloi* and *T. terrestre*. The feature which in our opinion most easily distinguishes the perfect stage of *A. curreyi* from those of *K. ajelloi* and *T. terrestre* is the presence in *A. curreyi* of peridial hyphae with a basic spiral configuration (pl. 3d). These spiral peridial hyphae are branched, usually to the outside of the spiral, and predominate particularly in young ascocarps. They can also be seen however with little difficulty in mature cleistothecia intermingled with straight or very slightly curved comb-like hyphae (pl. 3f) which are sparsely branched, usually dichotomously. We have been unable to confirm that anastomoses of peridial hyphae occur. The ascospores are lenticular in shape (pl. 3b) rather than ovoid as stated by Benjamin (1956) and Kuehn (1958) and do not have an equatorial groove as suggested by Kuehn.

### **Microsporum nanum** Fuentes

*M. nanum* was first described by Fuentes, Aboulafia & Vidal (1954) as *M. gypseum* var. *nana*. They isolated it in Cuba from ringworm lesions on the scalp of a boy and the body of an adult. In 1956, Fuentes published a valid description and gave the fungus specific rank. *M. nanum* has, as far as we know, been found on only one other occasion when it caused an outbreak of ringworm in pigs in Kenya. We obtained isolates from Mr. J. G. O'Sullivan, Glasgow

University Veterinary School and Mr. G. Pepin, Veterinary Laboratory, Weybridge each of whom had received material from Kenya. To confirm the identity of these cultures we obtained a Fuentes' isolate of *M. nanum* from Dr. Ajello. The isolates from Cuba and Kenya were cultured on soil but neither produced the perfect stage. Sexual reproduction occurred, however, when they were cultured together on baited unsterilised soil. Cleistothecia were also produced by certain crosses of single ascospore strains when they were mated in soil culture but never when they were cultured singly. This indicates that *M. nanum* is heterothallic. However sexual reproduction does not occur as readily or with the same frequency as in the other fungi studied and a number of single ascospore strains crossed with known plus and minus types have consistently failed to produce the sexual stage. Furthermore, in successful crosses the cleistothecia are produced rather sparsely and are usually deeply embedded in the vegetative mycelium. They mature slowly and the complete cycle from inoculation of compatible strains to ripening of cleistothecia takes 10–12 weeks at 24° C. The small number of cleistothecia formed and the long incubation period required for their production makes the study of sexual reproduction in this species exceedingly difficult. It is probable that the conditions provided are far from optimum and studies of the effect of variation of soil type and of environmental conditions are being made.

### **Microsporum gypseum** (Bodin) Guiart et Grigorakis

Full details of the perfect state of *M. gypseum* are given by Stockdale (1961), who has named it *Nannizzia incurvata*. We isolated *M. gypseum* on 4 occasions from soils in the West of Scotland and once from the hair of a clinically normal wild rat. Soil culture of the 5 isolates, in all possible combinations and of each isolate alone, failed to result in cleistothecial formation. The reason for these failures became apparent when, by mating these isolates with single ascospore strains of Stockdale's strain D.132, it was found that 4 of the isolates were of the same mating type and the fifth could not be induced to reproduce sexually. However, we recently received a number of soil samples, suspected to be the source of *M. gypseum* ringworm in a number of agricultural workers, from Dr. J. Alsop, Alcester and Dr. A. P. Prior, No. 14 Hospital Group Pathological Laboratory, Warwick, England. The isolates from these soils formed cleistothecia profusely on the hair-bait on the surface of the soil from which they were isolated and the cleistothecia are identical to those of *N. incurvata* Stockdale.

## Discussion

The exact conditions necessary to induce sexual reproduction in this group of fungi are unknown. However, it is clear that growth on agar media is in most instances quite unsatisfactory and the reasoning of Vanbreuseghem & Van Brussel (1952), which encouraged them to investigate soil culture in order to reproduce natural conditions as closely as possible, was sound. However, they sterilised the soil before use and we have found that unsterilised soil gives more satisfactory results. Possibly the presence of other soil organisms, either by their antagonistic or nutritional effects, stimulates sexual reproduction. The keratinaceous material used as bait is also important. Poor results were obtained with adult human hair or rabbit fur and we have found the most useful bait materials to be horse mane or tail hair, guinea-pig hair, cow-horn shavings, feathers and certain types of child's hair. Autoclaving of the materials before use does not affect the results.

Sexual reproduction is also affected by temperature and in the early stages of our work soil cultures were incubated at 28° C. which had been found satisfactory for cleistothecial formation in *K. ajelloi*. However, the study of *T. terrestre* showed that sexual reproduction was almost entirely suppressed by incubation at 28° C. and that excellent results were obtained at 24° C. Investigation of temperature effect on sexual reproduction in *M. nanum* showed that for this fungus also the most satisfactory results were obtained at the lower temperature.

Heterothallic species obviously require the meeting of both mating types before sexual reproduction can take place but it does not always follow that a culture containing compatible mating types will reproduce sexually even when substrate and temperature factors are advantageous. Most isolates of *T. terrestre* were potentially capable of sexual reproduction but required the stimulation of contact with single ascospore strains or isolates from another source before they did so.

The homothallic *A. curreyi* will form cleistothecia freely on most common agar media. However, as the time of conservation in artificial culture increases there is a concomitant decrease in the frequency with which sexual reproduction occurs and eventually only asexual spores are produced. Growth for a period in soil rejuvenates the fungus and sexual reproduction again occurs readily in artificial culture. We have induced cleistothecial formation by *K. ajelloi* on complete medium (Pontecorvo *et al.*, 1953) and by *T. terrestre* on complete medium and on Czapek-dox agar supplemented with 1 % peptone. However, the proportion of successful attempts was small and factors other than substrate and temperature are clearly important. It was found, for instance, that culture in test-tubes was vastly superior to that in Petri dishes and that an inoculum of mixed compatible strains usually failed to result in cleistothecial formation, whereas adjacent inoculation of the mating strains was reasonably satisfactory. It was noted that the peridia of cleistothecia formed on agar media were usually less well developed than those of cleistothecia formed in soil culture.

The cleistothecial initials of all these fungi consist of a club-shaped antheridium encompassed by a coiled ascogonium (pl. 1b). Spiral appendanges to the cleistothecium are also common to all of them although their number and size may vary considerably from cleistothecium to cleistothecium within the same species. For this reason these appendages are of little value in determination of species. However, their presence does indicate a relationship with the genera *Arthroderma* Currey, *Shanorella* Benjamin and the new genus *Nannizzia* Stockdale.

As has been stated, the characters given by Benjamin (1956) and Kuehn (1958) for the genus *Arthroderma* will allow the inclusion of the perfect states of *K. ajelloi* and *T. terrestre*. We therefore propose the name *A. uncinatum* for the perfect state of *K. ajelloi* to describe the uncinate branching of the peridial hyphae and *A. quadrifidum* for the perfect state of *T. terrestre* to describe the four-cleft nature of the mature cells of the peridium.

The perfect form of *M. nanum* most closely resembles the genera *Shanorella* and *Nannizzia*. It differs from *Shanorella* in that the peridial cells are strongly echinulate and do not disarticulate at maturity. The peridial cells are, however, strikingly similar to those of *Nannizzia* and in addition certain of the terminal cells bear the characteristic elongate straight appendages of that genus (Stockdale, 1961). Although in the cleistothecium of *M. nanum* the branching of the peridial hyphae is predominantly dichotomous, the verticillate form does occur and we therefore propose that the perfect state of *M. nanum* be included in the genus *Nannizzia* as *N. obtusa* to describe the obtuse angle which the branches form with their main axis.

### *Arthroderma uncinatum* sp. nov. (pl. 1)

Fungus heterothallus. *Cleistothecia* globosa, pallido-bubalina, 300–900 μ, med. 500 μ diam., appendiculis exclusis. *Peridium* 90–150 μ, med. 125 μ crassum. *Hyphae peridii* pallido-flavae, hyalinae, septatae, uncinatim ramosae, plerumque ad latus ab hypha principe remotius. Cellae crasso-parietales, summe echinulatae, mediae graciles, aequaliter utrimque globosae, 7–11 μ × 4–7 μ. *Appendicula;* (1.) hyphae septatae volutae, in longitudine numeroque spiralium inter se longe dissimiles, laterales aut terminales; (2.) macroconidia, parietibus laevibus, multiseptata, fusiformia, lateralia aut terminalia. *Asci* subglobosi, tenuibus parietibus, evanidi 5·4–7·2 μ × 4·9–6·3 μ, octospori. *Ascospori* hyalini, laevi aut tenuiter asperati, lenticulares, 2·3–2·7 μ × 1·4–1·8 μ, in concursu lutei. *Stat. conid. Keratinomyces ajelloi* Vanbreuseghem, 1952.

Heterothallic. *Cleistothecia* globose, pale buff, 300–900 μ, av. 500 μ diam., excluding appendages. *Peridium* 90–150 μ, av. 125 μ, thick. *Peridial hyphae* pale yellow, hyaline, septate, uncinately branched usually to one side, the outside, of the main hypha. Cells thick-walled, strongly echinulate, symmetrically dumb-bell shaped, 7–11 μ × 4–7 μ. *Appendages;* (1.) Septate spirals, which vary considerably in length and number of turns, lateral or terminal, (2.) smooth-walled multiseptate, fusiform macroconidia, lateral or terminal. *Asci* sub-globose, thin-walled, evanescent 5·4–7·2 μ × 4·9–6·3 μ, 8-spored. *Ascospores* hyaline, smooth or finely roughened, lenticular, 2·3–2·7 μ × 1·4–1·8 μ, yellow in mass.

4305 *Stat. conid. Keratinomyces ajelloi* Vanbreuseghem 1952.
4306
4566 *Habitat:* Soil.

### *Arthroderma quadrifidum* sp. nov. (pl. 2)

Fungus heterothallus. *Cleistothecia* globosa, pallido-bubalina, 400–700 μ, med. 580 μ diam., appendiculis exclusis. *Peridium* 80–135 μ, med. 103 μ crassum. *Hyphae peridii,* pallido-flavae, hyalinae, septatae, uncinatim ramosae plerumque ad latus ab hypha principe remotius. Cellae, crasso-parietales, summe echinulatae, novellae mediae graciles utrimque globosae, in aetate umeri brevis similes condylis insignibus et in uno modo latere formatis, 8–13 μ × 5–9 μ. *Appendicula;*

hyphae septatae volutae, in longitudine numeroque spiralium inter se longe dissimiles, terminales aut laterales. *Asci* subglobosi, parietibus tenuibus, evanidi, 4–6 μ × 3·5–5 μ, octospori. *Ascospori* hyalini, laevi aut tenuiter asperati, lenticulares, 1·8–2·7 μ × 0·9–1·8 μ, in concursu lutei.
*Stat. conid. Trichophyton terrestre* Durie & Frey, 1957.

Heterothallic. *Cleistothecia* globose, pale buff, 400–700 μ, av. 580 μ diam. excluding appendages. *Peridium* 80–135 μ, av. 103 μ thick. *Peridial hyphae* pale yellow, hyaline, septate, uncinately branched usually to one side, the outside, of the main hypha. Cells thick-walled, strongly echinulate, dumb-bell shaped when young, when mature resembling a short humerus bone with the condyles much accentuated and formed on one face only, 8–13 μ × 5–9 μ. *Appendages:* Septate spirals which vary considerably in length and number of turns, lateral or terminal. *Asci* sub-globose, thin-walled, evanescent, 4–6 μ × 3·5–5 μ, 8-spored. *Ascospores* hyaline, smooth or finely roughened, lenticular, 1·8–2·7 μ × 0·9–1·8 μ, yellow in mass.
*Stat. conid. Trichophyton terrestre* Durie & Frey (1957).
*Habitat:* Soil, hair of wild rat, horse.

***Nannizzia obtusa*** sp. nov. (pl. 4)

Fungus heterothallus. Cleistothecia globosa, pallido-bubalina, 250–450 μ diam., appendiculis exclusis. *Peridium* ca. 50 μ crassum. *Hyphae peridii* pallido-flavae, hyalinae, septatae, ramosae plerumque bifurcae, aliquando verticillatae, angulo inter ramum et hypham principem plerumque obtuso. Cellae crasso-parietales, echinulatae, cylindratae, 8–20 μ med. 13 μ × 4–7 μ, alinquando 1–2 modicis constrictionibus. *Appendicula;* (1.) Spiralia septata, parietibus laevibus, stricte voluta, lateralia aut terminalia; (2.) hyphae elongatae graciles septatae usque ad 450 μ longae, terminales. *Asci* subglobosi, tenuis parietibus, evanidi, 5·5–6·5 μ × 5–6 μ, octospori. *Ascospori,* hyalini, laevi aut tenuiter asperati, lenticulares 2·7–3·2 μ × 1·2–2 μ, in concursu lutei.
*Stat. conid. Microsporum nanum* Fuentes 1956.

Heterothallic. *Cleistothecia* globose, pale buff, 250–450 μ diam., excluding appendages. *Peridium* approx. 50 μ thick. *Peridial hyphae* pale yellow, hyaline, septate, branching mostly dichotomous, occasionally verticillate, angle between branch and main hypha usually obtuse. Cells thick-walled, echinulate, cylindrical, 8–20 μ av. 13 μ × 4–7 μ, may have 1 or 2 slight constrictions. *Appendages;* (1.) septate, smooth walled, tightly coiled spirals, lateral or terminal (2.) elongate slender, septate hyphae up to 450 μ in length, terminal. *Asci* sub-globose, thin walled, evanescent, 5·5–6·5 μ × 5–6 μ, 8-spored. *Ascospores* hyaline, smooth or finely roughened, lenticular, 2·7—3·2 μ × 1·2—2 μ, yellow in mass.
*Stat. conid. Microsporum nanum* Fuentes (1956).
*Habitat:* Isolates, from ringworm lesions in humans and in pigs, cultivated in soil.

Herbarium specimens of cleistothecia and cultures of compatible single ascospore strains of *A. uncinatum, A. quadrifidum* and *N. obtusa* have been deposited at the Commonwealth Mycological Institute, Kew, Surrey, England and the Centraalbureau voor Schimmelcultures, Baarn, Netherlands.

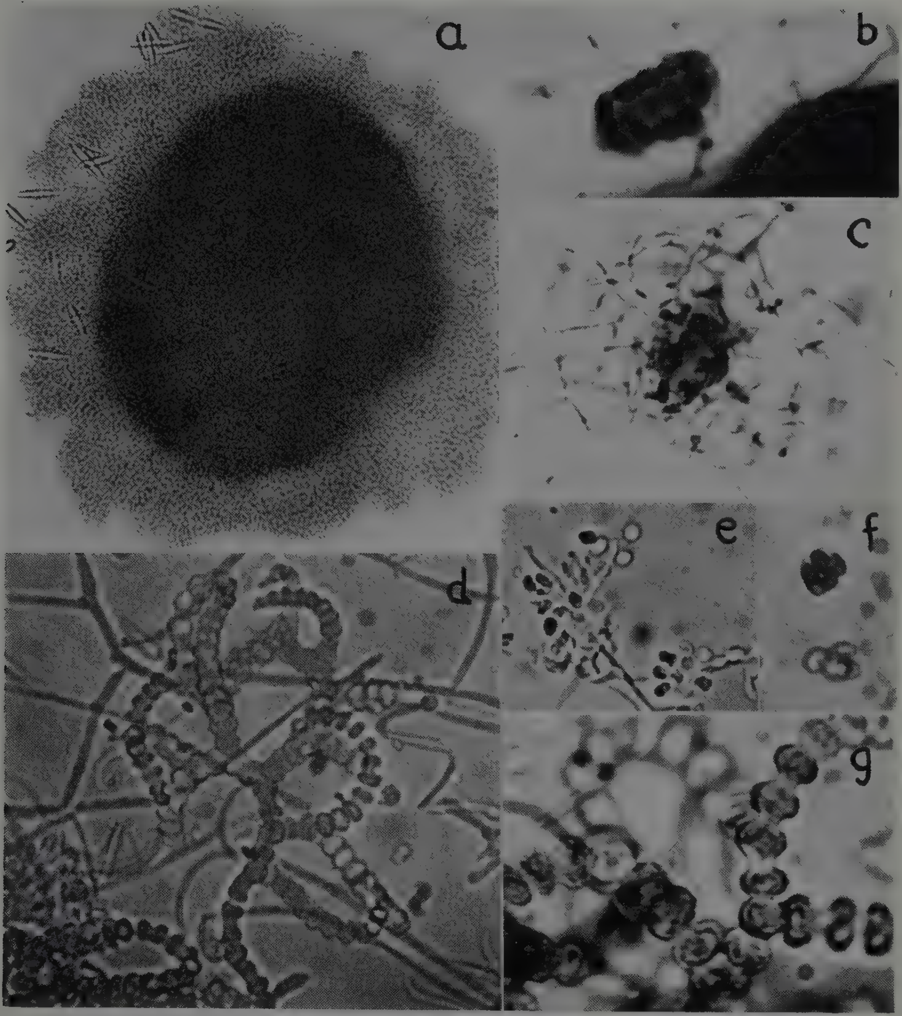

Plate 1.—*Arthroderma uncinatum a,* mature cleistothecium × 140; *b,* cleistothecial initials × 840; *c,* early stage of developing clesitothecium × 560; *d,* peridial hyphae showing uncinate branching × 560; *e,* ascogenous hyphae showing young asci × 560; *f,* ascus × 1400; *g,* dumb-bell shaped cells of peridium × 1400.

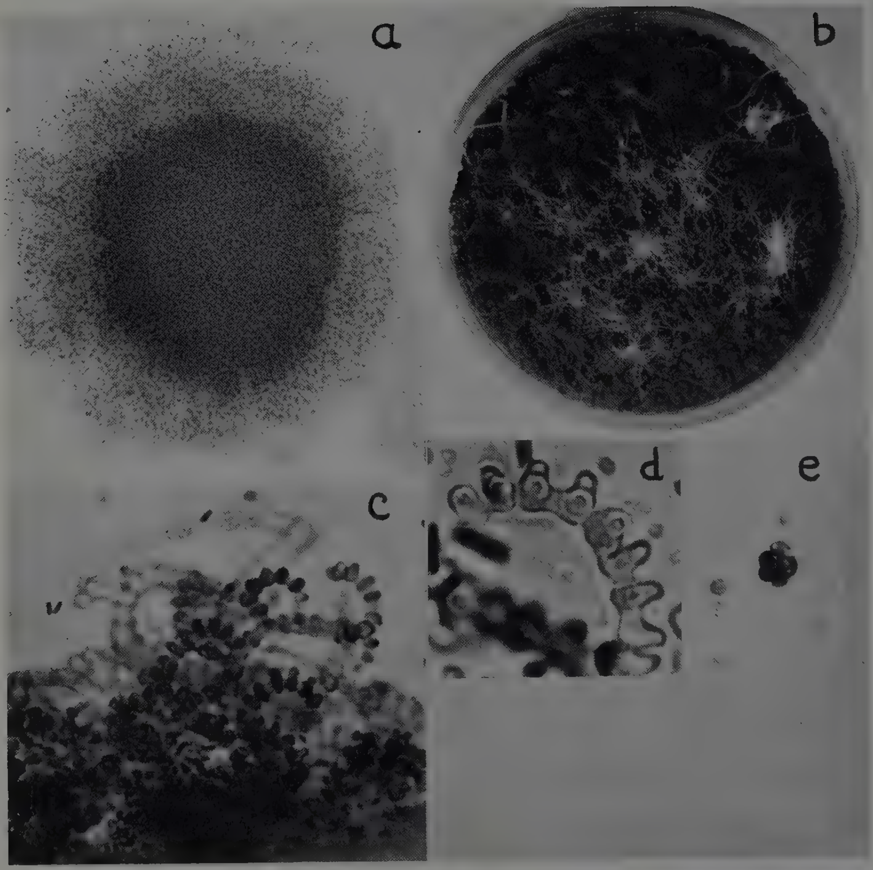

Plate 2.—*Arthroderma quadrifidum a*, mature cleistothecium × 140; *b*, cleistothecia in soil culture with guinea-pig hair bait; *c*, uncinately branched peridial hyphae × 560; *d*, distinctively shaped cells of peridium × 1400; *e*, ascus × 1400.

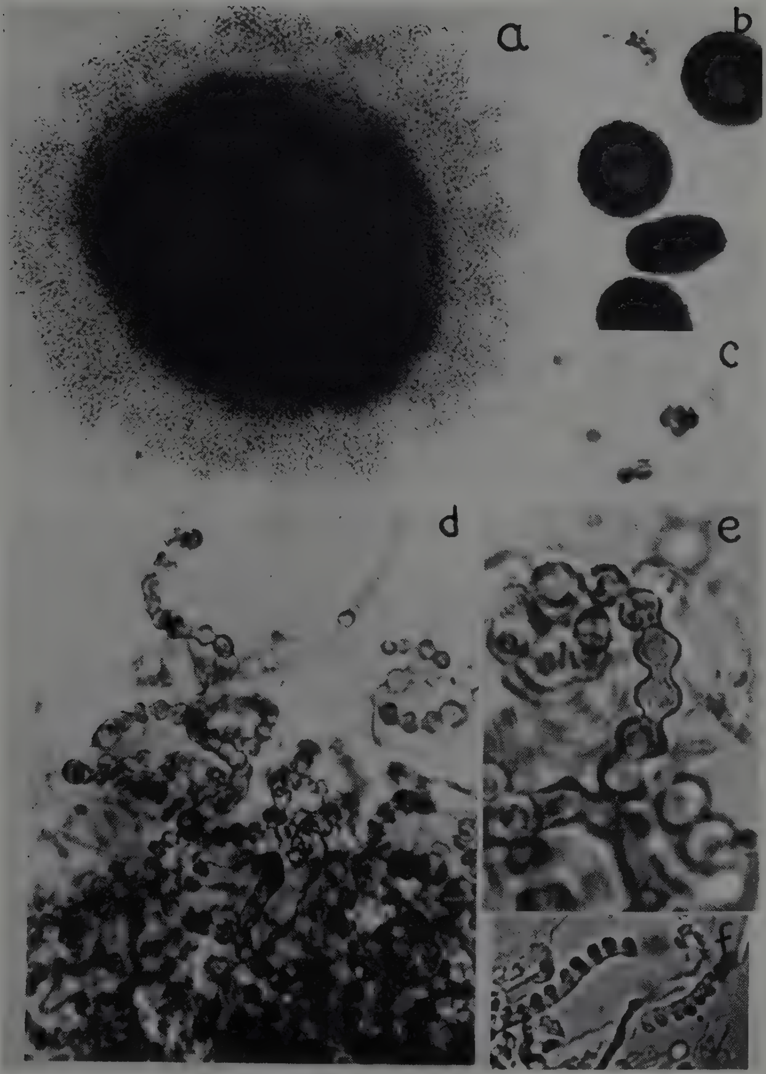

Plate 3.—*Arthroderma curreyi a*, mature cleistothecium × 140; *b*, electron micrograph of ascospores × 9,450; *c*, ascus × 1400; *d*, peridial hyphae showing spiral configuration × 560; *e*, dumb-bell shaped cells of peridium × 1400; *f*, comb-like hyphae of peridium × 560.

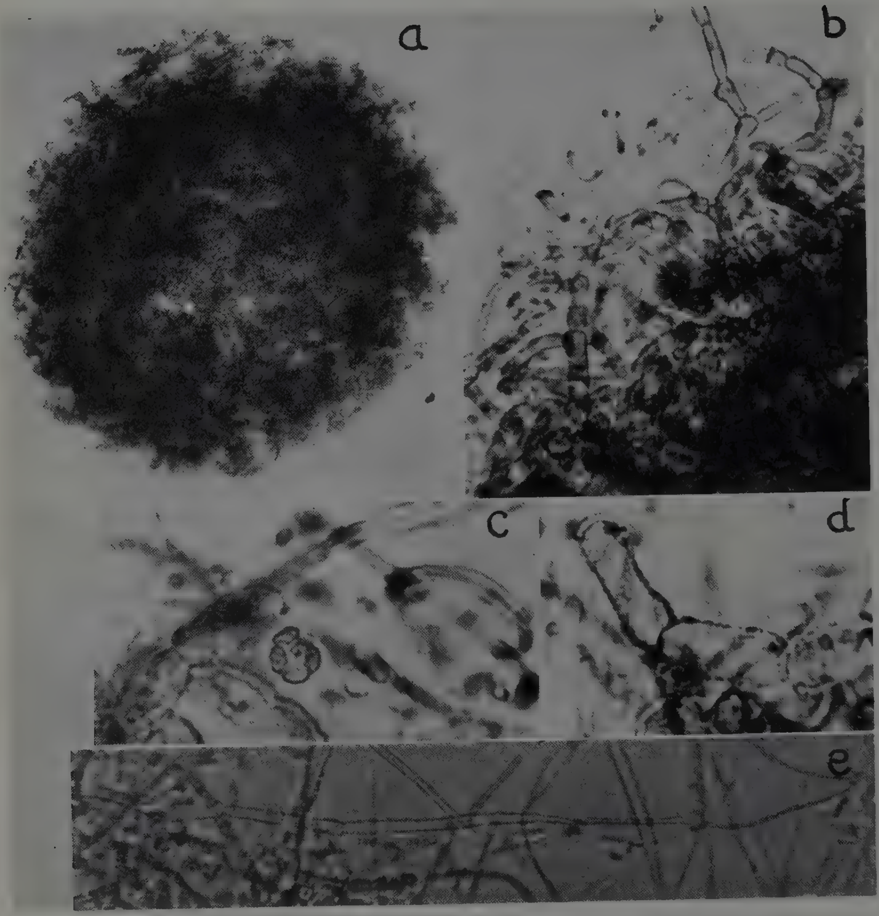

Plate 4.—*Nannizzia obtusa a*, mature cleistothecium × 140; *b*, peridial hyphae × 560; *c*, ascus × 1400; *d*, cells of peridium × 1400; *e*, long slender terminal appendage × 560.

## RÉSUMÉ

Au cours de recherches qui avaient pour but d'examiner des échantillons de sol provenant de plusieurs régions de l'ouest de l'Ecosse, en employant la méthode de culture sur cheveux de Vanbreuseghem (1952), on a isolé *Keratinomyces ajelloi, Trichophyton terrestre, Microsporum gypseum* et *Arthroderma curreyi.* Ces champignons, et *M. nanum* isolé de porcs infectés de teigne au Kenya, ont été étudiés en cultures sur sol non-stérilisé. On décrit les stades parfaits de *K. ajelloi* et *M. nanum* obtenus dans des cultures sur sol et également de *T. terrestre* lesquels se produisent spontanément chez une souche cultivée sur milieu gélosé. On propose d'inclure les formes parfaites de *K. ajelloi* et *T. terrestre* dans le genre *Arthroderma* Currey comme *A. uncinatum* sp. nov. et *A. quadrifidum* sp. nov. respectivement et que la forme parfaite de *M. nanum* soit incluse dans le genre *Nannizzia* Stockdale comme *N. obtusa* sp. nov.

## ACKNOWLEDGEMENTS

We are grateful to Mr. G. B. A. M. Finlayson and to Dr. H. R. Saunders for preparation of the Latin diagnoses and French summary respectively. We also wish to thank Dr. I. M. Dawson, Dr. J. Gordon and Mr. G. Mills for electron microscope studies, Miss E. Brown for technical assistance and those persons, mentioned in the text, who sent cultures for study.

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# THE EXTRA-HUMAN OCCURRENCE OF *TRICHOPHYTON TONSURANS* VAR. *SULFUREUM* IN A RESIDENTIAL SCHOOL

D. W. R. Mackenzie

*Department of Microbiology, Queen's University of Belfast.*

Following the discovery of *T. tonsurans* var. *sulfureum* at a Belfast residential school for girls, a series of mycological investigations was carried out to determine the extent to which the pathogen existed in non-living sites and to demonstrate the possible means of transmission from infected to non-infected children.

By the sampling techniques adopted the pathogen was isolated on 89 occasions, 52 of these being from hair brushes or combs. Infection was also found on the surfaces of pillowslips and other bedclothes, floors and a curtain. It was demonstrated in the atmosphere in the vicinity of infected children's beds during bedmaking. Adoption by the school staff of simple hygienic precautions and a more rigid supervision of the manner in which toilet articles were used resulted in a marked suppression of extra-human infection.

Among 128 girls in a Belfast residential school clinical infections with *T. tonsurans* var. *sulfureum** were found in 21 children ranging in age from 6–13 years. Infection took the form of *tinea capitis* in 14, *tinea corporis* in 4 and *tinea capitis* et *corporis* or *unguium* in 3. The clinical details and the attempts made to eradicate infection by general hygienic measures and griseofulvin treatment have been described by Mackenzie, Burrows and Walby (1960). In this paper details of the mycological investigations are presented, with particular reference to the occurrence of the fungus in extra-human sites.

## Methods

Since most of the infected children appeared to have ringworm of the scalp, initial searches for the extra-human occurrence of the fungus were made at sites such as the surfaces of pillows, portions of bedspreads overlying pillows, individual hairbrushes and combs, hair clippers, towels and floors. Subsequent examinations were made at sites where the fungus might not be suspected, such as laundered and stored clothing, furniture, curtains, mattresses, lockers, personal effects, etc. (table 1).

Atmospheric sampling was carried out in an attempt to demonstrate if infection could be transmitted aerially. It seemed reasonable to suppose that if pillows, sheets or bedspreads were carrying infection, the fungus could be disseminated by bedmaking. The choice of sites was again largely selective and atmospheric sampling was carried out at the sides of beds belonging to children known to be infected. In later studies, however, a number of settling plates were distributed at random throughout the school.

*Medium:* All isolations were made in petri dishes containing 12–15 ml. 2·5% malt agar incorporating penicillin (20 units/ml.), streptomycin (40 units/ml.) and cycloheximide ('Actidione') (0·5 mg./ml.).

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* This name appears never to have been validated. *Trichophyton tonsurans* Malmsten var. *sulfureum* (Sabouraud) comb. nov. (syn. *T. sulfureum* Sab., *Les Teignes*, 1910, p. 317) is therefore proposed.

*Sampling:* Atmospheric sampling was carried out by means of a 'Castella' slit sampler with pre-set sampling times of 1, 2 or 5 min., and an intake of 1 cu. ft. (approx. 28·6 l.) of air/min.

Beds were made by the children who were encouraged to adopt methods normally used. Mutual assistance was common and since it was found that approximately 2 min. were required for routine bedmaking, this time was selected for sampling. Prior to bedmaking an initial control plate was exposed for 5 min. in the slit sampler and this was immediately followed by a second exposure (2 min.) taken whilst the bed was being made. Thirty minutes later a third plate was exposed for 5 min. Throughout each test the slit sampling apparatus remained in one position.

Direct sampling was possible with hairbrushes and combs. The bristles of the brushes or the teeth of the combs were pressed several times into the medium, to give a multiple inoculation of the surface (fig. 1). Different areas of each brush or comb were inoculated in turn thus ensuring that the distal portions of most of the bristles or teeth were brought into contact with the surface of the agar.

Where direct inoculation of the agar surface was not possible (*e.g.* with clothing or bedding), samples were made by means of sterile gauze pads, approximately 6·5 cm. square, placed aseptically on the agar surface of plates and stored at 4° C. until required. Samples were obtained by removing the gauze pad from the petri dish with the fingers and rubbing vigorously over the site under examination. On plane surfaces an area corresponding to 1 sq. ft. (approx. 0·1 sq. m.) was sampled. The pad was then pressed firmly on to the agar surface and the plates incubated at 26° C. After 48 hours the gauze squares were removed with sterile forceps and discarded and the plates incubated for an additional 12 days. Colonies of *T. tonsurans* var. *sulfureum* appeared 5 to 12 days after inoculation of the plates. Great care was exercised during sampling to reduce the possibilities of accidental contamination.

This method was designed to accommodate the large numbers of samples envisaged and was adopted because of its simplicity and the ease with which sampling could be made. Unlike the other techniques used the method is qualitative rather than quantitative, *i.e.,* results indicate the presence of the fungus at any particular site rather than the amount present. Samples of dust from cracks in floors or walls where the gauze pad method was impracticable were taken with bacteriological swabs.

## RESULTS

The results obtained are summarized in table 1.

*Bathrooms:* Brushes and combs were stored in communal bathrooms each of which served 2 or 3 dormitories. They were housed individually in a series of open-fronted compartments or pigeon-holes which had been constructed throughout of wood, painted, and clamped to the wall.

During the first visit to the school, samples were taken at 1 bathroom used by 48 children; at this time only 5 girls were known to be infected, but it was found that no fewer than 17 hairbrushes or combs gave rise to colonies of *T. tonsurans* var. *sulfureum* (table 2). Owing to a re-allocation of compartments it was not possible to establish ownership of the infected brushes or combs.

Furthermore, although brushes or combs had originally been allocated to individual children, it was found that indiscriminate use of these articles was common.

Thirty-five other brushes or combs were found to be infected, and 30 (85·7 %) belonged to children who were known or ultimately shown to harbour infection.

| *Source* | *No. of samples* | *No. of positives* |
|---|---|---|
| Brushes/combs | 600 | 52 |
| Pillowslips | 292 | 19 |
| Floors | 31 | 4 |
| Atmosphere | 23 | 4 |
| Internal pillowslips | 26 | 2 |
| Sheets | 27 | 2 |
| Mattress covers | 25 | 1 |
| Mattresses | 25 | 1 |
| Bedspreads | 24 | 1 |
| Blankets | 24 | 1 |
| Curtains | 11 | 1 |
| Inside of locker | 35 | 1 |
| Other bedding | 23 | — |
| Towels | 36 | — |
| Clothing | 55 | — |
| Furniture | 79 | — |
| Others | 52 | — |
| TOTAL | 1,388 | 89 |

TABLE 1.—EXTRA-HUMAN SOURCES EXAMINED FOR THE PRESENCE OF *T. tonsurans* var. *sulfureum.*

With the exception of 1 child who had infection of the glabrous skin only, all 30 infected brushes or combs belonged to children with ringworm of the scalp.

After the initial visit, the staff of the school made an attempt to eradicate extra-human infection. It was later discovered that all hair brushes and combs had been treated with a proprietary black coal tar disinfectant, and a 'strong' solution of this had also been used to wash the individual compartments in which the articles were housed. A similar disinfection followed the second visit. Knowledge of these procedures was not obtained for several weeks. Although the strength of antiseptic employed was not known, its liberal application together with a more rigid insistence that each child used only her own article appeared to have a marked depressive effect on the number of positive samples (table 2).

|  | *1st visit 28.7.59* | *2nd visit 7.8.59* | *3rd visit 21.8.59* | *4th visit 11.3.60* |
|---|---|---|---|---|
| No. of articles examined | 49 | 91 | 93 | 100 |
| No. positive | 17 | 11 | 8 | 1 |
| % age positive | 34·7 | 12 | 8·6 | 1 |

TABLE 2.—ISOLATIONS OF *T. tonsurans* VAR. *sulfureum* FROM BRUSHES AND COMBS ON FOUR DIFFERENT VISITS.

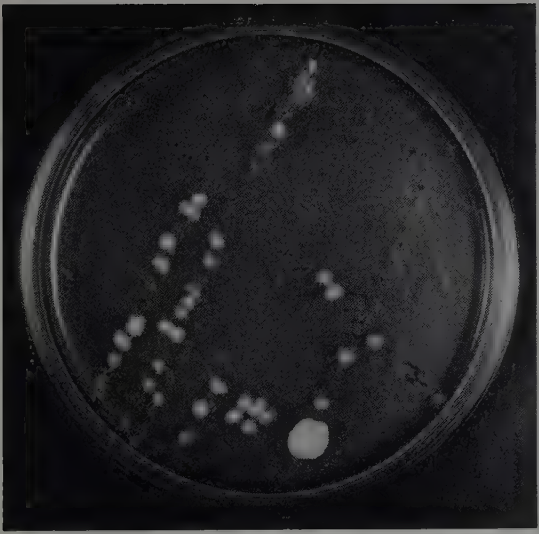

Figure 1.—Result of direct inoculation of the teeth of an infected comb on to an agar plate. Numerous colonies of *T. tonsurans* var. *sulfureum* are appearing at the points of inoculation.

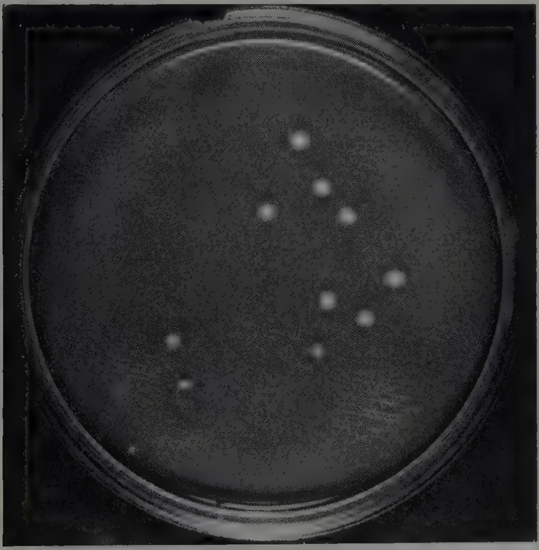

Figure 2.—Colonies of *T. tonsurans* var. *sulfureum* obtained from an infected child's pillowslip by sampling with a sterile gauze swab.

(*To face page* 60

*Pillowslips:* Of the 19 pillowslips shown to carry infection, 11 (58 %) were from beds of children who were known or later shown to be infected. Fig. 2 shows the result of sampling from the pillowslip of an infected child and table 3 the results obtained in successive visits to dormitories.

| | *1st visit*<br>*28.7.59* | *2nd visit*<br>*7.8.59* | *3rd visit*<br>*27.8.59* | *subsequent*<br>*visits* |
|---|---|---|---|---|
| No. examined | 29 | 43 | 98 | 122 |
| No. positive | 11 | 7 | 1 | 0 |
| % age positive | 38 | 16·3 | 1 | 0 |

TABLE 3.—ISOLATIONS OF *T. tonsurans* VAR. *sulfureum* FROM PILLOWSLIPS.

*Atmosphere:* Four out of 20 samples yielded *T. tonsurans* var. *sulfureum.*

(a) Settling plates: One plate, exposed for 20 min. on the floor of a dormitory where there were 6 infected children, gave rise to a single colony of the fungus.

(b) Slit sampling: Atmospheric samplings were made at 3 separate sites and on each occasion the findings were identical. The pathogen was not recovered during pre- or post-bedmaking samples, but was isolated each time during the 2 min. bedmaking sample.

*Floors: T. tonsurans* var. *sulfureum* was isolated from floors on 4 separate occasions. Three of the isolates were from the same dormitory floor and in each case from a portion adjacent to beds belonging to infected children. One of the colonies obtained from a gauze pad originated from a short (3 mm.) portion of human hair, and this was the only occasion on which the nature of the infective particle could be determined.

On the fourth occasion when the fungus was obtained from floors it was isolated by direct sampling from the bristles of a floor polisher.

*Others:* A single isolate was obtained from the curtains in 1 dormitory. Other sources yielding *T. tonsurans* var. *sulfureum* were various portions of bedding, including sheets and internal pillowslips (2 occasions each), a pillow, bedspread, blanket, mattress cover and mattress.

Sampling of the contents of a locker belonging to 1 infected child showed that *T. tonsurans* var. *sulfureum* was present, but although sampling was repeated on the next visit no further evidence of infection or its carrier could be obtained. It was noted, however, that this locker contained a pair of woollen knitted slippers and it was possible that these had picked up some infected material from the floor.

## DISCUSSION

The detection of pathogenic fungi in non-living sites can provide valuable evidence on the methods by which transmission can occur. Walker (1955) reported the presence of hairs infected with *T. discoides* (*T. verrucosum* var. *discoides*) on scratching posts used by cattle and suggested that infection of humans could occur from such extra-human sources. Gentles (1956) isolated

*T. mentagrophytes* and *T. rubrum* from the floors of bathhouses and in a later paper (Gentles, 1957) concluded, from a study of clean and used shower-house floors, that spread of the fungus probably followed transfer of infected skin fragments.

Adamson & Annan (1949) and Ajello & Getz (1954) isolated *T. mentagrophytes* from wash houses, and this fungus has also been obtained from the floor of a schoolhouse (Bocobo & Curtis, 1958) and the soil of caves (Lurie & Borok, 1955). An interesting record of the indirect isolation of *T. mentagrophytes* from the atmosphere was made by Lurie and Way (1957) who recovered the fungus from the lungs of animals exposed to the atmosphere of a cave for 5 hr. So far as is known the isolation of *T. tonsurans* var. *sulfureum* in the present studies constitutes the first record of direct isolation of a dermatophyte from the atmosphere.

Schwarz, Peck, Botvinick, Liebovitz & Frasier (1946) found *Microsporum canis* on hairdressing instruments and considered that barbers' shops were important in the spread of ringworm during epidemics. Failure in the present study to detect infection on the hair clippers used to trim the children's hair was rather unexpected. Since over 13 % of the children in the school had a scalp infection, the hair clippers could legitimately be suspected of spreading ringworm. It was found that the clippers were rinsed after use in a dilute solution of the coal tar disinfectant and failure to isolate the fungus can perhaps be attributed more to efficient disinfection than to limitations of sampling technique.

Indirect transmission of ringworm fungi will occur only if (1) the fungus is viable and capable of remaining so for a considerable period when separated from its host, (2) susceptible hosts come into contact with the sites bearing infective material, and (3) conditions favouring the establishment of infection are present (*e.g.* mild trauma may be necessary). Ringworm fungi are known to remain viable for long periods after being shed from the parent lesions. Strains of *T. equinum* for example can persist for up to 7 years (Stcherbatykh, 1956). Little is known of the ability of *T. tonsurans* var. *sulfureum* to exist in its parasitic form apart from its host but reisolations have been made for up to 11 months from infected material stored in folded notepaper at room temperature in this laboratory. *T. tonsurans* var. *sulfureum* had almost certainly been present in the school for 4 years prior to the present investigations (Mackenzie, Burrows & Walby, 1960). Since 21 children were known to be infected at the end of that period, the amount of fungus-infected material deposited in the school was likely to be high, and this was confirmed by the large numbers of isolations during the earlier visits. The total number of affected children after a 4-year period represented only 16.4 % of the population of the school and this suggests that *T. tonsurans* var. *sulfureum* possesses a comparatively low degree of infectivity.

On the first visit the high number of fungal isolates obtained from hairbrushes and combs apparently indicated a high incidence of human infection. However, the marked reduction in numbers of isolates following the adoption and enforcement of simple hygienic precautions and a more strict supervision of the allocation and use of these articles showed that this was apparent rather than real. Nevertheless, brushes were used more or less indiscriminately by the children at first, and in view of the heavy infection detected on several of these articles, this practice was probably responsible for bringing about considerable spread of the fungus.

The significance of pillowslips and bedding as reservoirs of infection and the the role they play in spread of the disease is not easily determined. Transmission of viable fungus could conceivably follow contact between a child and an infected sheet or pillowslip during play or in the making of beds and atmospheric sampling shows that the movements of bedclothes are sufficient to distribute infected particles.

Routine bedmaking, naturally occurring air currents and the movements of children about the dormitory could be responsible for the distribution of considerable quantities of potentially pathogenic material such as arthrospores, portions of infected hairs or epidermal scales; dust, loose fibres of cotton and similar materials could act as carriers of the fungus. The only evidence of the nature of the pathogenic particles was obtained in a plate inoculated with a floor swab, where a colony was seen to originate from a small portion of hair. Microscopic examination revealed that fragments of human hair were abundant in the material collected from floors and window ledges, although infected hairs were never observed. The reduction in the number of positive samples obtained from hair brushes and combs at successive visits (table 2) was even more pronounced in the samples from pillowslips (table 3). Here, the number of positive samples obtained over a 4½ week period dropped from 38 % to 1 %, this marked reduction being the outcome of more frequent laundering and replacement of all pillowslips. The suppression of communal usage of hair brushes and combs presumably also had an effect in reducing the contamination of pillowslips.

Aerial spread of the organism could account for its isolation from floors, and could also account for its recovery from a curtain, whilst isolates made from bedding other than pillowslips could be the result of deposition of infective material from an active lesion or spread during bedmaking, or both.

## CONCLUSIONS

The techniques employed and the results obtained have served to show that *T. tonsurans* var. *sulfureum* was present in non-living sites, and that spread of infective material could occur during bedmaking or by normally occurring air currents. Evidence of the mode of spread must necessarily be circumstantial, but factors which can be considered contributory include the indiscriminate use of hair brushes or combs and bedmaking. Other factors which will contribute towards spread of infection include the ability of the pathogen to remain viable when isolated from its host and the presence in the school for some years of infected children. Many opportunities for contact between the fungus and susceptible individuals must arise from the gregarious nature of life at a residential school.

## ACKNOWLEDGEMENTS

Acknowledgement is made to the Supervisor and staff of the school for permission to carry out sampling experiments, Miss Maura McArdle for technical and practical assistance and Mr. R. G. Wood for photography.

## ZUSAMMENFASSUNG

Nach Feststellung von *T. tonsurans* var. *sulfureum* in einer Internatschule für Mädchen in Belfast wurde eine Reihe von mykologischen Untersuchungen vorgenommen, um festzustellen, zu welchem Grad dieser Pilz auf leblosen Gegenständen vorhanden war, und um zu zeigen, wie er von angesteckten Kindern auf nicht angesteckte übertragen werden kannte.

Durch die zu diesem Zweck angewandten Probemethoden wurde der Pilz 89 Mal festgestellt, worunter 52 Mal auf Haarbürsten und Kämmen. Der Organismus wurde auch auf den Oberflächen von Kissenüberzügen und anderem Bettzeug, Fußboden sowie einem Vorhang gefunden. Er wurde auch beim Bettmachen in der Luft nahe den von angesteckten Kindern benutzten Betten festgestellt. Die Anwendung von einfachen hygienischen Schutzmaßnahmen und eine strengere Aufsicht über den Gebrauch von Toilettensachen führten zu einer erheblichen Verminderung an außermenschilcher Ansteckung.

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# OBITUARIES

## HUBERT JAUSION, M.D.

1890-1959

Hubert Jausion died at his home in Paris on the 7th Oct. 1959. He was held in high esteem in French dermatological circles and was the author of more than 500 papers and a textbook, 'Les Maladies de Lumière'. He was an active member of some 15 medical and scientific societies, including the Association of Medical Mycologists of France, and was a Past President of several of these. At the time of his death he was head of the laboratories at the Franco Musulman Hospital in Paris and had recently been Professor agrégé du Val de Grace. His publications are chiefly concerned with dermatology, particularly the immunology of diseases caused by light, and with medical mycology. In the latter field he made several contributions concerning the clinical aspects of ringworm, particularly favus, and on the clinical effect of fungous extracts, which he called mycoclasines, on various pathological conditions. Although the number of his publications is large, they are outnumbered by his many fields of interest and by the number of topics on which he could speak with ease.

Dr. Jausion served in the first world war and was awarded the Croix de Guerre and the Legion d'Honneur for gallantry.

Everyone who met Hubert Jausion will recall his amazing memory, his wide knowledge of French literature, his sharp wit and good-natured sense of fun. He had a flair for poetry and made good use of this to express his approval of man or deed. The distinction of his large circle of friends gives a clear reflection of this many-talented man whose loss is deeply and most sincerely regretted.

The International Society for Human and Animal Mycology expresses its deep sympathy to his widow.

R. VANBREUSEGHEM.

## JEAN PIERRE BERNAERTS, M.D.

1928–1960

Jean Pierre Bernaerts, who, because of his youth, was unknown to many members of the ISHAM, died on the 16th Feb. 1960 at the early age of thirty-two.

He graduated from the Université Libre de Bruxelles in 1953 and was awarded a grant from the Belgian Ministère des Colonies to study at the Prince Léopold Institute for Tropical Medicine at Antwerp in order to prepare himself for work in the Belgian Congo. It was in my laboratory at the Institute that his first experimental work, dealing with the production of grains in laboratory animals infected with *Allescheria boydii* and *Monosporium apiospermum* was carried out.

At the end of his stay at the Prince Léopold Institute, the diagnosis of an incurable illness put an end to his hope of a career in the tropics. Aided by his devoted wife, who is also a physician, this charming young man was able to turn from research work and to establish a successful private practice in Brussels, as though he had never envisaged anything else.

I visited him several times during his stay in hospital and I can vouch for the fact that his courage and keen interest in medicine and mycology never failed.

To Mrs. Bernaerts and his two young children the International Society for Human and Animal Mycology expresses its very sincere sympathy.

R. VANBREUSEGHEM.

# REVIEWS

*Mycological Research : Proceedings of the Fifth Leningrad Mycological Conference.* Edited by KASCHKIN, P. N., ARAVYSKY, A. H. & KOKUSHINA, T. M. (Kirov State Institute for Further Education. Leningrad, 1960). Pp. 308. In Russian. English summary,

This collection of 47 papers presented at the Fifth Mycological Conference at Leningrad in December, 1959 deals with candidiasis, dermatomycoses and some systemic mycoses. The 20 papers on *Candida* spp. cover the morphological, biochemical, immunological, pathological and epidemiological aspects and also treatment. There are 9 contributions on the dermatomycoses including one on onychomycosis. Epidermophytosis in children is described by Samarina, Kushneva and Novikova and the role of bath-house floors in its spread is mentioned. The paper on chronic trichophytosis in adult males due to *Trichophyton violaceum* is concerned with the source of infection. From the results of studies of *T. sulphureum* and *T. crateriforme* (*tonsurans*) Bakhareva concludes that the species are synonymous. In the papers on treatment of dermatomycoses there is no mention of griseofulvin.

A fungus resembling *Keratinomyces ajelloi* was isolated by Daletzkaya from soil in the Leningrad region using Vanbreuseghem's technique.

Coccidioidomycosis is discussed in 8 papers. Only a few cases of this condition have been diagnosed, and there are indications that the fungus may differ from that encountered in U.S.A. With isolates from the U.S.S.R. arthrospores (chlamydospores?) are very rarely formed in culture and spherules are seldom seen in inoculated laboratory animals. A number of other systemic mycoses which occur only rarely are dealt with, *viz.* rhinosporidiosis, maduromycosis and chromoblastomycosis.

It is indicated that future research in medical mycology should deal particularly with epidemiology and treatment.

J. G. O'S.

*Diagnostic de Laboratoire en Mycologie Médical.* (Laboratory Diagnosis in Medical Mycology). G. Segrétain, E. Drouhet & F. Mariat. (Éditions de la Tourelle, St. Mandé (Seine) 1958) Pp. 140, 25 figs.

This useful little book gives the elements of the laboratory techniques in medical mycology in a form which can be easily understood. It deals first briefly with elementary general mycology and with methods of microscopy and culture of pathological material. The various mycoses and their causal fungi are then dealt with in turn and for each, special techniques for study and the form of the fungus in tissue and in culture are given. The last few pages deal with preparation of mountants, stains and media, and a few illustrations of common saprophytes and a short glossary are given. General references only are included.

J. C. G.

*Laboratory Identification of Pathogenic Fungi Simplified.* Elizabeth L. Hazen & F. C. Reed. (Blackwell Scientific Publications Ltd., Oxford, England, 1960). 2nd Edition. Pp. 150. £3.

The first edition of this useful practical manual appeared in 1955 and the production now of a second edition is a clear indication of its value. The general layout is as before, with two main sections, one dealing with the superficial mycoses and the other with the systemic mycoses and sections on aspergillosis and mucormycosis have been added. The new edition also deals with species of *Candida* other than *C. albicans* and includes a small section on common contaminants. The high standard of the illustrations, set in the first edition, has been maintained.

J. C. G.

*Das kulturell-biochemische und serologische Verhalten der Cryptococcus-Gruppe.* (The cultural, biochemical and serological behaviour of the Cryptococcus group). Seeliger, H. P. R. *Ergebn. Immunforsch.* **32,** 23–72 (8 fig., 204 ref.) 1959.

In this most useful monograph on the Cryptococcus group the history, nomenclature and classification are reviewed and details of the author's researches on the cultural, biochemical and serological behaviour of *Cryptococcus* and related non-pathogenic genera are given. As a result of his work the author states that *Torulopsis aeria* belongs to the genus *Cryptococcus.* However, despite certain morphological and biochemical characteristics in common with *Cryptococcus, Lipomyces* retains its generic status and the author finds no evidence to substantiate the hypothesis that *C. neoformans* is the imperfect state of *L. starkei.* It is stated that differentiation of *Cryptococci* from other yeasts is possible in 24–96 hours by cultural, biochemical, morphological and serological tests but to distinguish *C. neoformans* from other members of the genus inoculation of laboratory animals is necessary.

C. O. D.

*Mykosen der Haut* (Mycoses of the skin). Kalkoff, K. W. & Janke, D. in *Dermatologie und Venerologie* Gottron, H. A. & Schonfeld (George Thiene, Stuttgart, 1958). Vol. 2, pt. 2, p. 991 – 1153. 85 fig.

This well illustrated monograph deals, in a short introduction, with the fundamentals of mycology and immunology. There follows a detailed account of the etiologic agents, epidemiology, pathogenesis, symptomatology, serology and therapy of the cutaneous mycoses including sporotrichosis and those occasional infections caused by common saprophytic moulds. Some infections which are usually called 'mycotic' although not caused by true fungi *e.g.*, erythrasma, actinomycosis, nocardiosis, and trichomycosis palmelliarum are also discussed in detail. The cutaneous forms of the systemic mycoses (histoplasmosis, coccidioidomycosis, North and South American blastomycoses) are not dealt with.

H. P. R. S.

*Manuale di Micologia Medica* (Handbook of Medical Mycology) R. Ciferri (Renzo Cortina, Pavia). Vol. 1, Pp. 370, 304 figs. 1958, Vol. 2. Pp. 976, 605 figs. 15 col. pl. 1960. U.S. $32.

Volume 1, a general introduction to medical mycology, begins with a historical review of the subject and this is followed by a brief treatment of nomenclature and classification of the mycoses and of the fungi. The general aspects of the fungi indicating their importance as saprophytes, as parasites of plants and animals and as allergens, are then given briefly. Their physiology, morphology and taxonomy, with particular reference to those important to the medical mycologist, are dealt with in some detail. The techniques of microscopy and culture and a brief introduction to serology and immunology as applied to the fungi are followed by short sections on inoculation of laboratory animals and their subsequent investigation by biopsy and culture. Therapy of mycotic infections and presentation of results of studies in a form suitable for publication are given in the last chapter. Two keys, one on diagnosis of the mycoses and the other on identification of fungi are included. Volume 2 deals in detail with all the known mycoses and each disease is given the same logical treatment, *viz.* definition of the disease, geographical distribution, hosts, causal fungi, mode of infection, clinical symptoms, examination of pathological material, culture, histology, animal inoculation, prognosis and therapy. There are also brief sections on the fungi as allergens and on fungi and algae which are occasional pathogens. Both volumes are well illustrated and the illustrations are of a high standard. The author has clearly made considerable effort to consult all available references and it is regrettable that the bibliographs which are intended to be a feature of the work are incomplete. It is pointed out that, because of the expense of printing, a number of references have been omitted but this is of little satisfaction to the reader who, after searching the various sections, finds that the particular reference he is seeking is missing. Some system of abbreviated entry would have avoided this difficulty. In a number of instances the spelling of the authors' names or the dates of the references given in the text differ from those given in the bibliograph. In both volumes the bibliographs are collected at the back of the book and one feels that in volume 2 they would have been more accessible at the end of the relevant chapter. Despite these deficiencies however, the work constitutes a most useful and up-to-date contribution to medical mycology. The text throughout is clear and carefully written and sufficient emphasis is given to both clinical and mycological aspects to render the books well suited for use by clinician and mycologist alike. A useful and comprehensive index to both volumes is given in volume 2. The binding is pleasing and of a robust nature and the type is clear and distinct.

J. C. G.

## Films

*Étude biologique et anatamo-pathologique du Pied de Madura* [Produced by the Institut Pasteur in collaboration with affiliated institutes overseas.]

This film deals with the history of Madura Foot, its epidemiology in tropical Africa and with investigations of clinical, histological, pathological and radiological aspects. The principal causal organisms, the microscopic characters of the grains and the results of extensive cultural studies are also dealt with. The film concludes with a summary of the therapeutic measures employed. It is in colour, 16 mm., with a sound commentary in French and runs for 20 minutes. Applications to purchase the film or to obtain on loan should be made to Institut Pasteur, 25 rue du Docteur Roux, Paris XV, France.

*The Griseofulvin Story*

[Produced by Glaxo Laboratories Ltd., England.]

Produced in 1959 for medical and scientific audiences this film deals with the discovery of griseofulvin in 1939 and traces the various stages in its development until it was accepted in 1959 as a therapeutic agent for the treatment of dermatomycoses. Some detail is given of the large-scale fermentation methods of production and of the agricultural uses. The film is in colour, 16 mm., with a sound commentary in English and runs for 21 minutes. It is available on loan by application to the Public Relations Service, Glaxo Laboratories Ltd., Greenford, Middlesex, England.

## Formation of New Medical Mycology Society

The growing awareness of the importance of the mycoses in medicine is illustrated by the formation of a new medical mycology society in Germany. It was founded on 31st May 1960 during a meeting in the Academy of Medical Education, Berlin, attended by Professor Friedeberger the Deputy Minister of Health in the German Democratic Republic. Professor Braun, Director of the Skin Clinic of the Medical Academy, Magdeburg, and Professor Wildführ, Director of the Institute of Medical Microbiology and Epidemiology, Leipzig, were elected as joint chairmen and Dr. Langer as secretary. A provisional programme was prepared and it was agreed that a meeting of all members and a paper-reading session would be held in Berlin during late November 1960. The topic for discussion was 'The perspective of Mycology in the German Democratic Republic as viewed by the dermatologist, the microbiologist and the veterinarian'. Applications for membership should be made to the Secretary, Dr. Langer, Hautklinik der Charite der Humboldt-Universität, Berlin N.4, Schumannstr. 21.

Contributed.

## Society News

The 8th Internationl Congress of Microbiology will be held at Montreal, Canada during 19-25 August 1962. It is understood that one session of the congress will be devoted to a symposium on a topic in the field of medical and veterinary mycology. It is hoped that it will be possible to arrange a meeting of the Society at Montreal in August 1962. Details will be announced as soon as possible.

Members are reminded that subscriptions ($3.00 or equivalent in other currencies) for 1961 are now due and should be paid as soon as possible to the General Secretary, account number 133.700, Banque d'Anvers, Meir 48, Antwerp, Belgium.

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## INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

### INCOME AND EXPENDITURE ACCOUNTS

*For the period 1/9/58 – 31/12/58**

| RECEIPTS | | PAYMENTS | |
|---|---|---|---|
| Balance in hand at 1st September 1958 | 10,747:07 | Bank charges | 167:00 |
| Subscriptions | 8,159:33 | | |
| Interest (Bank of Antwerp) | 145:00 | Balance at 31st Dec. 1958 | 18,885:00 |
| Belgian Francs | 19,052:00 | Belgian Francs | 19,052:00 |

* The accounts of the society were submitted to and passed by an elected committee during the society meeting in Lisbon, September 1958.

*For the year ending 31/12/59*

| RECEIPTS | | PAYMENTS | | |
|---|---|---|---|---|
| Balance in hand at 1st January 1959 | 18,885 | Administrative costs (including those for last 4 months of 1958) | | 28,848 |
| Subscriptions | 52,642 | Bank charges | | 218 |
| Interest (Bank of Antwerp) | 654 | Printing and Publishing:— | | |
| Donation from Robert Werner | 300,000 | Graphica—Stationery | 2,363 | |
| | | Goemaere—4th Bulletin | 13,684 | |
| | | | | 16,047 |
| | | Subscription, Federation of International Associations (Belgium) | | 100 |
| | | Editor's expenses | | 5,025 |
| | | Balance at 31st December 1959 | | 321,943 |
| Belgian Francs | 372,181 | Belgian Francs | | 372,181 |

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# METABOLIC STUDIES OF THE TISSUE PHASE OF *CANDIDA ALBICANS* INDUCED *IN VITRO*

CLAIRE L. TASCHDJIAN and PHILIP J. KOZINN*

*Department of Pediatrics, Maimonides Hospital,*
*Brooklyn, New York*

The tissue morphology of *Candida albicans,* characterized by ovoid thinwalled blastospores with small vacuoles and by mycelia, was reproduced *in vitro* on dialysates of sera and plasmas.

On these substrates *C. albicans* utilized all available amino acids as well as glucose without evolution of carbon dioxide and without appreciably changing the pH. Sulfhydryl was not liberated, and Triphenyltetrazolium chloride (TTC) was not reduced by budding or filamentizing spores, or by hyphae. Similar results were obtained when 3% serum albumin was added to the substrate.

In whole serum, and in dialysates containing gamma globulin, budding was suppressed and no free amino acids were utilized although all available glucose was consumed.

Serum proteins in whole serum and serum albumin and/or gamma globulin added to dialysates were not broken down.

TTC was not reduced by the mycelial phase of *C. albicans* in serum, or in mouse tissue upon inoculation by the airpouch technique.

The tissue morphology of *C. albicans* could not be reproduced on amino acid standards in aqueous solutions approximating the composition and concentration of free plasma amino acids and on this substrate amino acid uptake was totally or partially inhibited.

In the presence of M/100 cysteine in serum and serum dialysates *C. albicans* maintained the spherical thickwalled yeast morphology characteristic of growth on artificial culture media and budded freely with liberation of sulfhydryl into the substrate.

Failure of the induced tissue phase of *C. albicans* to liberate sulfhydryl and to reduce TTC are interpreted as indications that cell division *in vivo* involves enzymatic pathways that differ from those known to be operative *in vitro.*

Maintenance of the tissue morphology appears to be related to utilization of all free serum amino acids in the presence of an unidentified, dialysable serum factor. This factor is inhibited in serum, but is apparently present in tissues that favour infection with *C. albicans.*

Failure of *C. albicans* to break down serum proteins and the apparent relationship between tissue morphology and amino acid utilization are taken to indicate that *C. albicans in vivo* may also subsist on free amino acids without breaking down tissue protein. Such free, watersoluble amino acids have been demonstrated in freshly formed keratin.

These considerations imply that interference with keratinization may be one factor in the host-parasite interaction in superficial candidiasis.

Factors influencing growth, cell division, and dissociation into yeastlike and mycelial forms in *Candida albicans* have been studied extensively *in vitro.* Little is known about conditions that influence these phenomena *in vivo* as part of the environment furnished by the host in the host-parasite interaction in candidiasis.

Nickerson and his coworkers showed in various studies that glucose and a high intracellular level of sulfhydryl are prerequisites for maintenance of the yeast phase (Y-phase) *in vitro.* Glucose provides a readily metabolized substrate which enables *Candida* cells enzymatically to reduce intracellular disulfide

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* This study was supported by United States Public Health Service Grant E-1751, and by grants from the Squibb Institute for Medical Research and the Jewish Philanthropic League of Brooklyn.

(cystine or glutathione). Cysteine may be substituted effectively for glucose, both for the maintenance of cell division and for reversal of the mycelial phase (M-phase) to the Y-phase (Nickerson & Mankowski, 1953; Nickerson & Chung, 1954; Winsten & Murray, 1956).

Nickerson & Chung (1954) found that growing cells of *C. albicans* liberate extracellular sulfhydryl, probably cysteine, which may be demonstrated by reduction of bismuthylhydroxypolysulfite in the culture medium.

Maintenance of the Y-phase *in vitro* entails transport of hydrogen from a metal component of the flavoprotein (probably Zn or Fe) to the cell division enzyme (a disulfide reductase) (Nickerson, 1954; Nickerson & Falcone, 1956; Ward & Nickerson, 1958). Blocking of hydrogen transport to this enzyme can be demonstrated by the reduction of tetrazolium salts to colored formazans by the "spillover" hydrogen, and results morphologically in conversion of Y to M.

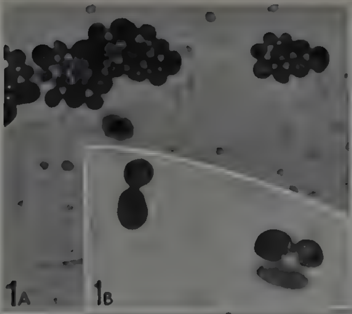
It has been shown previously that the Y-phase of *C. albicans* is associated *in vivo* with saprophytism, and that the appearance of M coincides with invasion of tissues and clinical signs of candidiasis (Taschdjian & Kozinn, 1957; Taschdjian, Reiss & Kozinn, 1960). In typical *Candida* lesions, Y and M are present in similar proportions. In monilial granuloma M predominate to such an extent that the histopathologic picture suggests dermatophytosis rather than candidiasis (Hauser & Rothman, 1950; Pluss & Kadas, 1954). Almost immediate conversions of Y to M may be induced experimentally by inoculation of *C. albicans* into serum (Taschdjian, Kozinn & Burchall, 1960), or into mice by Selye's airpouch technique (Hill & Gebhardt, 1956).

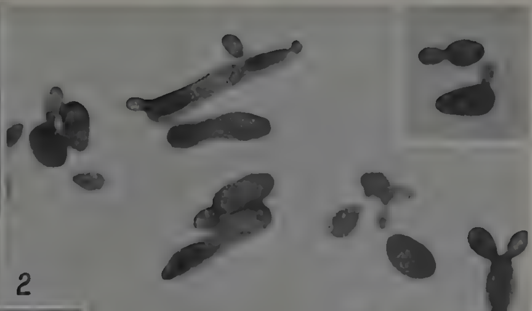
These facts suggest that *C. albicans* subsists saprophytically in human hosts under conditions that favour cell division. Invasion of the host's tissues is associated with at least partial inhibition of the cell division process, resulting in the presence of both M and Y.

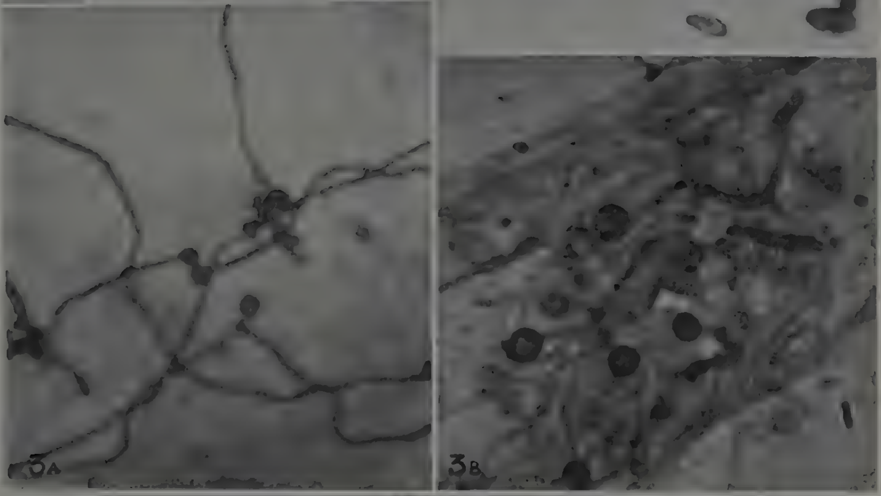
If the conditions that influence morphology and morphogenesis in *C. albicans in vivo* correspond to those *in vitro,* one would expect M/Y to liberate sulfhydryl, and M to reduce TTC (triphenyltetrazolium chloride). A basic study of these phenoma is possible only if the tissue morphology of *C. albicans* can be reproduced *in vitro.*

Attention has not hitherto been called to the fact that the Y morphology of *C. albicans* seen *in vivo* differs from that seen *in vitro.* On conventional culture media (such as Sabouraud's agar), the spores of *C. albicans* are spherical and thickwalled and have large vacuoles. The axis of the bud is continuous with that of the mother cell (fig. 1). In lesions, the spores are ovoid, thinwalled, and have small vacuoles. Buds are produced at an angle to the mother cell (fig. 2). For convenience, this *in vivo* morphology will be called TY ("Tissue Yeast"), and the yeast shape seen in cultures will be referred to as CY ("Culture Yeast").

The marked difference between the morphology of *C. albicans* in serum (fig. 3) and that seen in tissues (fig. 2) indicates differences in the metabolism of the organism in these two environments. It is logical to assume that components of the blood that enter the tissues furnish part of this environment *in vivo,* and play a part in the maintenance of the tissue morphology (TY/M).







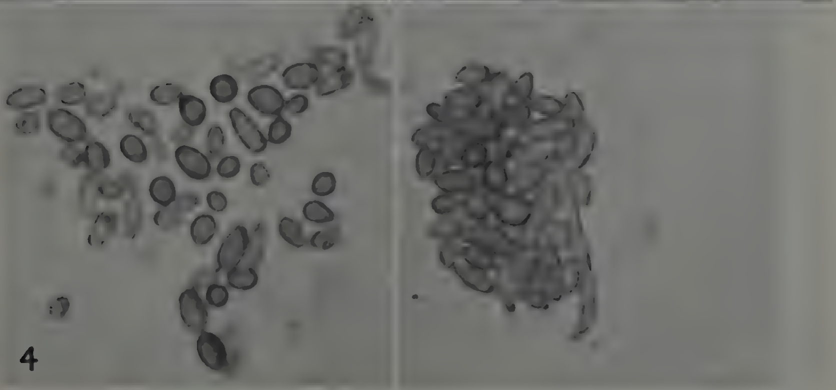


Figure 1.—Morphology of *Candida albicans* on Sabouraud's agar, 48 hours, PAS stain. (A) × 450. (B) × 3600.

Figure 2.—Morphology of *Candida albicans* spores in a direct smear from oral thrush. PAS stain, × 3600.

Figure 3.—Morphology of *Candida albicans* seen in serum (A), and in airpouch inoculation of mice (B), each after 48 hours' growth. Note large, thickwalled cells giving rise to filaments, and absence of budding. A similar morphology has been noted in monilial granuloma lesions.

Figure 4.—Morphology of *Candida albicans* on serum dialysate, 48 hours' growth. PAS stain × 450. Note conversion to ovoid, thinwalled TY morphology with small vacuoles.

Foremost among these components are the free amino acids from which tissue protein is presumably synthesized (Fleischer, Lietze, Walter & Haurowitz, 1959).

Dialysates of serum and plasma were therefore used as substrates in the present attempt to reproduce the tissue morphology of *C. albicans* and to study amino acid and glucose uptake of the organism under simulated *in vivo* conditions.

Since each step in these experiments was determined by the results obtained in the preceding step the methods used and results obtained will be presented consecutively.

## METHODS, RESULTS AND COMMENT

### *1. Induction of Tissue Morphology of C. albicans on Serum Dialysates*

Pooled adult and newborn (cord) sera were dialysed under sterile conditions against 5-10 times their volume of distilled water. *C. albicans* was grown in the dialysates either during dialysis at 37°C., or after dialysis at 4°C. Concentration of dialysates of fresh sera to the original serum volume resulted in inhibition of *Candida* growth by the dialysable antifungal factor described by Lorincz, Priestley & Jacob (1958) and by Roth, Boyd, Sagami & Blank (1959). This factor is unstable and was no longer operative in dialysates from stored blood-bank plasmas which were used in the experiments described under 3a.

Samples of *Candida* were withdrawn at intervals for microscopic observation and compared with parallel growths maintained on saline and on serum.

The dialysate cultures grew well. Conversion to the tissue morphology occurred within 6 to 8 hours. After 24-48 hours' incubation TY predominated over M. (fig. 4). This morphology was maintained for 96 hours, until the substrate was nutritionally exhausted.

On saline, *C. albicans* remained in the non-budding resting stage. On serum, M were formed within 2-4 hours and the M-phase persisted throughout the period of incubation (fig. 3).

Transfer of centrifuged and washed M-phase from serum to serum dialysate resulted in prompt conversion to and maintenance of TY/M, and *vice versa*.

Swabs from oral thrush lesions retained their tissue morphology upon inoculation into serum dialysate but converted to M upon direct inoculation into serum. These results are illustrated diagramatically in fig. 5.

The dialysable components of serum thus appeared to provide an environment similar to that encountered by *C. albicans in vivo,* as implied by the prompt conversion of the organism to the tissue morphology, regardless of the source of the inoculum.

### *2. Amino Acid and Glucose Uptake of the "Tissue Phase" of Candida Albicans*

Utilization by *C. albicans* of amino acids and of glucose after 5 days' growth on serum dialysates was studied by means of 1 and 2-dimensional paper chromatography. After concentration of substrates and blank controls to 1/2 to 1/5 of the original serum volume, 10 µl. of each sample were chromatographed. The methods used were essentially those of McMenamy, Lund & Oncley (1957), Levy & Chung (1953) and Wright, Ullstrom & Szewczak (1957).

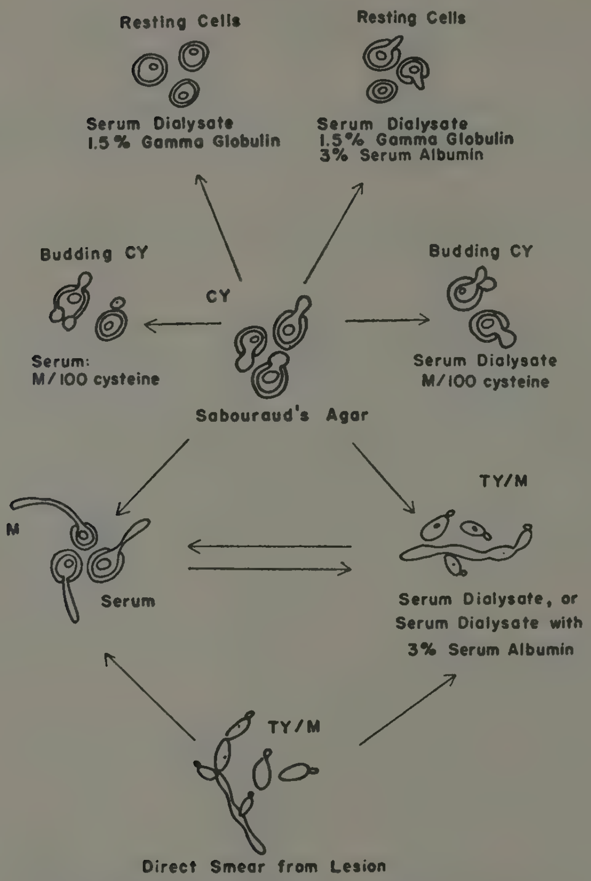

Resting Cells
Serum Dialysate
1.5 % Gamma Globulin
Resting Cells
Serum Dialysate
1.5% Gamma Globulin
3% Serum Albumin
Budding CY
Serum:
M/100 cysteine
CY
Budding CY
Serum Dialysate
M/100 cysteine
Sabouraud's Agar
M
Serum
TY/M
Serum Dialysate, or
Serum Dialysate with
3% Serum Albumin
TY/M
Direct Smear from Lesion

Figure 5.—Diagram illustrating morphologic response of *Candida albicans* Culture phase (CY) and Tissue phase (TY/M) upon transfer to serum and serum dialysates.

The following ninhydrin- and isatin-reactive substances were identified in blank serum dialysates: alanine, glutamine, glutamic acid, glycine, histidine, isoleucine, lysine, proline, serine, threonine, and valine. Methionine and phenylalanine were visualized occasionally. Arginine, alpha-amino-n-butyric acid, taurine and tyrosine, reported by McMenamy *et al.* (1957) as present in plasma dialysates in small amounts, could not be demonstrated.

All amino acids contained in the dialysates were fully utilized by *C. albicans* within 5 days and were no longer demonstrable by chromatography. Urea levels, determined by staining the chromatograms with Ehrlich's reagent, remained unchanged. No catabolic products were recognized by the methods used after the comparatively brief period of incubation required for nutritional depletion of the substrate.

Glucose was no longer demonstrable after 12-24 hours growth. An unidentified reducing sugar noted by Wright *et al.* (1957) in newborn serum was not metabolized by *C. albicans.*

These findings confirmed the visual impression that the dialysable components of serum provided an excellent medium for *C. albicans*. No difference was noted in that respect between adult and newborn sera.

### *3. Factors Influencing Serum Morphology and Tissue Morphology of C. albicans*

#### *(a) Serum Proteins*

Dialysates of outdated bloodbank plasmas were used in these experiments, since the dialysable antifungal factor described by Lorincz *et al.* (1958) and by Roth *et al.* (1959) was no longer operative. This permitted concentration of the dialysates to the original serum volume. These dialysates differed from those of fresh sera only in lack of glutamine. They were as satisfactory for induction of TY/M as were dialysates of fresh sera.

It was sought to determine whether the presence of gamma globulin, or of serum albumin, or of both serum proteins, inhibited conversion of CY to TY/M and was responsible for the M morphology seen in the whole serum.

Serum albumin (3%) and gamma globulin (1·5%) were added separately and together to plasma dialysates concentrated to the original plasma volume. In cultures containing gamma globulin growth was poor. *C. albicans* remained predominantly in the non-budding resting stage and did not assume the M-morphology characterizing growth on serum. In the presence of serum albumin alone growth was excellent. Conversion to TY/M occurred within 4 hours. Chromatograms showed that all available amino acids were fully utilized within 5 days.

Electrophoresis and total protein determinations showed that the serum proteins were not broken down in either whole serum, or in dialysates to which they had been added separately or together. The free amino acids were not utilized in any of the substrates that contained gamma globulin. Glucose was metabolized on all substrates.

These results indicate that conversion to the tissue morphology is inhibited by non-dialysable serum components other than serum albumin. Inhibition appears to be associated with blocking of amino acid utilization but does not extend to the uptake of glucose. *C. albicans* reacts morphologically in two ways to this inhibition. Utilizing available glucose but not amino acids it retains the resting stage in the presence of gamma globulin in serum dialysates, while on serum it reverts to the M-phase. Gamma globulin, therefore, does not seem to be the only inhibitory factor involved.

#### *(b) Amino Acids*

The results obtained thus far indicated that the tissue morphology of *C. albicans* may be associated with utilization of free amino acids. It was therefore attempted to induce the tissue morphology on aqueous solutions containing the dialysable plasma amino acids in the concentrations used by McMenamy *et al.* (1957) and in the present studies as chromatographic standards[1]. These amino acid solutions were used with and without addition of 0·1 % glucose. Serum and serum dialysates served as controls.

Morphologically, the results obtained on the synthetic substrates were intermediate between those seen on serum (M) and on serum dialysates (TY/M). Both M and Y were present. The Y shape was ovoid, resembling TY, but the vacuoles were larger, corresponding in size to those seen in CY.

Glucose was again utilized fully within 24 hours. Amino acid uptake was partly or totally inhibited. In the presence of glucose none of the amino acids in the synthetic substrate was utilized. In the absence of glucose only glutamic acid, glycine, histidine, proline and threonine were metabolized.

Conversion to, and maintenance of, the tissue morphology of *C. albicans* seems to be related to utilization of all available free amino acids. Some, as yet unidentified, dialysable component of serum appears to be required by *C. albicans* for this purpose. The activity of this factor appears to be inhibited in serum, but not in the tissues.

### *(c) Fermentation and pH*

Although glucose was consumed rapidly there was no evolution of $CO_2$ in serum and in serum dialysates during one week's incubation. The initial serum pH was not changed; the pH of the serum dialysates shifted slightly from 8·25 to 7·4.

An alkaline pH and a low concentration of reducing sugars are known to favour Y → M conversion *in vitro* (Teissier, 1890; Langeron & Guerra, 1939; Johnson, 1954; Morton, 1955). Serum and serum dialysate are very similar as regards glucose content and pH, yet M predominate in serum and Y prevail in the dialysates. *In vivo,* Y are associated with saprophytism and M/Y with parasitism (Taschdjian & Kozinn, 1957; Rogers, 1957), under presumably similar conditions of pH and glucose supply in the oral cavity and the gastro-intestinal tract. It therefore seems doubtful that these 2 factors affect morphogenesis and morphology of *C. albicans in vivo.*

## *4. Enzymatic Activities of the "Tissue Phase" of Candida Albicans*

### *(a) Liberation of Sulfhydryl, and Reaction to Cysteine*

Extracellular liberation of sulfhydryl was tested by means of bismuthylhydroxypolysulfite* as previously reported by Nickerson & Chung (1954). In order to separate the "-SH trap" from the liquid substrate the bismuth compound was incorporated into a melted 2 % agar solution in distilled water and this was caused to form a thin coating on the inner wall of the test tubes by rotation while the agar solidified.

Serum, plasma dialysate, and plasma dialysate with 3 % serum albumin, were used and parallel cultures were kept on Sabouraud's agar. On the serum substrates the cultures remained grossly and microscopically colorless and the bismuth compound was not reduced. It appeared, therefore, that *C. albicans* did not liberate sulfhydryl from serum albumin which contains 80% of the total serum -SH according to Weissman, Schoenbach & Armistead (1950). On Sabouraud's agar the colonies showed a brownish-black discoloration but no blacking of the culture medium, indicating extracellular "leakage" of -SH, was observed.

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[1] Milligrams per 100 ml of distilled water: alanine 7·66, arginine 3·9, glutamic acid 5·0, glycine 5·2, histidine 5·2, isoleucine 2·6, leucine 4·0, lysine 6·4, methionine 3·7, phenylalanine 2·6, proline 5·6, serine 3·6, threonine 5·2, tryptophan 3·0, valine 7·0.

* The authors are grateful to Dr. A. Kupferberg and the Ortho Research Foundation for a supply of bismuthylhydroxypolysulfite.

A strong reaction was observed, however, when M/100 cysteine was added to the serum substrates, There was intense blacking of the bismuth-agar film, obviously due to activity on the part of *C. albicans,* since this reaction did not occur in corresponding blank substrates kept as controls.

In the presence of cysteine M were suppressed, both in serum and in the dialysates, and *C. albicans* retained the *in vitro* morphology of budding CY. This observation agrees with Nickerson's findings (1953, 1954) that cysteine stimulates cell division but indicates also that cysteine may not be a factor that influences cell division and morphology of *C. albicans in vivo*. In this connection it is interesting to note that cysteine was not found among the free dialysable amino acids of blood plasma (McMenamy *et al.,* 1957), among the free amino acids occurring on the human skin surface (Rothman & Sullivan, 1949; Frankel & Reiches, 1961), in freshly formed keratin (Szakall, 1955) or in vaginal secretions (Gregoire, Lang & Ward, 1959), *i.e.,* in the cutaneous and mucocutaneous sites that favour infection with *C. albicans.*

### *(b) Reduction of TTC (Triphenyltetrazolium Chloride)*

If M formation of *C. albicans in vivo* results from blocking of hydrogen transport to the cell division enzyme as demonstrated by Ward & Nickerson (1958) on artificial culture media, liberation of hydrogen should be demonstrable also under *in vivo* conditions by reduction of TTC to a red formazan within the *Candida* cells. TTC (1%) was added to serum and serum dialysates, and samples were withdrawn for microscopic observation at 24 hour intervals for 1 week.

After 1 week's incubation the cultures were macroscopically pink. Microscopically reduced TTC was noted only within resting cells. Budding cells, cells giving rise to M, and M, did not reduce the tetrazolium salt.

A suspension of *Candida albicans* in 1% aqueous TTC solution was injected into mice by the airpouch technique described by Hill & Gebhardt (1956). Examination of the mouse tissue after 24 hours revealed complete conversion of *C. albicans* to the M phase but TTC was not reduced by the organism or by the surrounding host tissues.

## Discussion and Conclusions

The results obtained in the present studies indicate that *in vivo* cell division and inhibition of cell division occur under conditions that differ extrinsically and intrinsically from those known to influence these processes *in vitro*. Among extrinsic factors that are operative *in vitro* but not *in vivo* are pH and glucose concentration of the substrate. As regards intrinsic factors, failure of the "tissue phase" of *C. albicans* to liberate sulfhydryl and of the M phase to reduce TTC, implies enzymatic pathways of cell division *in vivo* that differ from the flavoprotein-disulfide reductase pathway demonstrated by Nickerson and his coworkers *in vitro* (1953, 1954, 1956, 1958). At present the nature of these pathways is not known. However, the present studies indicate that utilization of free amino acids may be associated with the intrinsic mechanisms that regulate cell division *in vivo* and enable *C. albicans* to assume and maintain its tissue morphology. These enzymatic pathways appear to be inhibited by certain non-dialysable serum components. Such an inhibition is only partly evident in those tissues that are commonly infected with *C. albicans,* permitting the existence of both M and Y forms.

The question arises whether *C. albicans* possesses proteolytic enzymes which enable it to invade tissues and to satisfy its amino acid requirements by the breakdown of tissue proteins. Breakdown of keratin by *C. albicans in vitro* has been reported by Kapica & Blank (1957) after 40-50 days' incubation and in the presence of much larger concentrations of glucose than are present under physiologic conditions. On the other hand, it is extremely difficult to produce *Candida* infection of intact human skin (Maybach, 1960), or even to achieve invasion of excised tissues (Beemer, Pryce & Riddell, 1954).

The observations presented here have shown that *C. albicans* is unable to break down serum proteins. On the other hand, the free dialysable amino acids of serum provided a favourable medium for growth and maintenance of the tissue morphology. These same amino acids have been demonstrated on the normal skin surface (Rothman & Sullivan, 1949; Frankel & Reiches, 1961), in freshly formed keratin (Szakall, 1955), and in vaginal secretions (Gregoire *et al.,* 1959), *i.e.* in specimens from sites that are susceptible to *Candida* infection. These considerations point to the possibility that *C. albicans in vivo* may subsist on free amino acids, thus depriving the host of raw material for the synthesis of protein, notably keratin. This assumption is supported by well-known clinical phenomena such as scaling, exfoliation and denudation of cutaneous and mucocutaneous lesions, and crumbling and discoloration of the proximal and lateral portions of the nail in onychia.

Interference with keratinization may therefore be one factor in the host-parasite relationship in superficial candidiasis, a possibility that is now under investigation.

## ZUSAMMENFASSUNG

Die Gewebsmorphologie von *Candida albicans,* charakterisiert durch oval-dünnwandige Blastosporen mit kleinen Vakuolen und Myzel, wurde *in vitro* auf Serum-Plasma-Dialysaten reproduziert. Auf diesen Substraten verbraucht *C. albicans* ohne Entwicklung von $CO_2$ und ohne wesentliche Änderung des pH's alle verfügbaren Aminosäuren und die Dextrose. SH wurde nicht freigesetzt; desgleichen wurde TTC weder durch die sprossenden und fadenbildenden Sporen noch durch die Hyphen reduziert. Ähnliche Ergebnisse wurden nach Zusatz von 3% Serum-Albumin zum Substrat erhalten. Im Vollserum und in Dialysaten mit Gammaglobulin war die Sproßung unterdrückt. Obwohl alle verfügbare Dextrose verbraucht wurde, wurden freie Aminosäuren nicht angegriffen. Die Serumproteine im Vollserum sowie Serum-Albumin und/oder Gammaglobulin, das den Dialysaten zugesetzt wurde, wurden nicht zerlegt. TTC wurde im Serum durch die Myzelphase von *C. albicans* nicht reduziert, ebensowenig im Mäusegewebe nach Impfung mittels der "airpouch"-Technik. Die Gewebsmorphologie von *C. albicans* ließ sich in wässrigen Aminosäure-Lösungen, deren Zusammensetzung und Kon-

zentration den freien Plasma-Aminosäuren weitgehend ähnelten, nicht reproduzieren. Die Aminosäureaufnahme war ganz oder teilweise gehemmt. In Gegenwart von M/100 Cystein im Serum und Serum-Dialysaten behielt *C. albicans* die sphärische, dickwandige Hefe-Morphologie, die charakteristisch für das Wachstum auf künstlichen Nährböden ist. Dabei kam es zu reichlicher Sproβung und Freisetzung von SH in das Substrat. Das Unvermögen zur Entwicklung von SH und zur Reduktion von TTC wird bei der induzierten Gewebsphase von *C. albicans* als Anzeichen dafür gewertet, daβ die Zellteilung *in vivo* auf enzymatischen Bahnen abläuft, die von denen *in vitro* verschieden sind. Die Erhaltung der Gewebsmorphologie scheint an den Verbrauch aller freien Aminosäuren in Gegenwart eines nicht identifizierten, dialysierbaren Serumfaktors gebunden zu sein. Dieser Faktor wird im Serum gehemmt, ist aber wahrscheinlich in Geweben vorhanden, die eine Infektion mit *C. albicans* begünstigen. Das Unvermögen von *C. albicans* zur Zerlegung der Serumproteine und die augenscheinliche Beziehung zwischen Gewebsmorphologie und Aminosäureverbrauch deuten darauf hin, daβ *C. albicans in vivo* von freien Aminosäuren ohne Spaltung von Gewebeprotein lebt. Solche freien wasserlöslichen Aminosäuren sind im frischgebildeten Keratin nachgewiesen worden. Aus diesen Überlegungen ergibt sich, daβ bei oberflächlicher Candidiasis Störungen der Keratinisierung einen Faktor bei den Wechselbeziehungen zwischen Gast und Wirt darstellen dürften.

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# STUDIES ON THE OCCURRENCE OF *HISTOPLASMA CAPSULATUM* IN PERU

Libero Ajello*, Alfred S. Lazarus†,
Alberto Cornejo‡ & Jane C. Moore*

* *Communicable Disease Center, U.S. Public Health Service, Department of Health, Education, and Welfare, Atlanta 22, Georgia.*
† *U.S. Operations Mission, c/o American Embassy, El Salvador, San Salvador.*
‡ *Universidad Nacional Mayor de San Marcos, Lima, Peru.*

Soil studies were carried out in the Peruvian Departments of Huánuco, Loreto, Cusco and Madre de Dios. *Histoplasma capsulatum* was recovered from 5 different soil samples collected in the village of Tingo María and its surrounding area (Huánuco). One soil sample, from the village of Quincemil in the Department of Cusco, also yielded that fungus.

The dermatophyte, *Microsporum gypseum*, was present in soils gathered in the Departments of Huánuco, Loreto and Cusco.

In 1954, ecological and epidemiological studies carried out in the village of Tingo María, Peru, resulted in the discovery of an unsuspected endemic area for *Histoplasma capsulatum*. This fungus was recovered from soil gathered in "La Cueva de las Lechuzas", a large cave populated by a colony of oil-birds (*Steatornis caripensis*) (Lazarus & Ajello, 1955). Shortly thereafter, the development of "Tingo María Fever" in 2 individuals, who had visited the oil-bird cave, was proven by culture to have been caused by *H. capsulatum* (Celso Arellano & Galvez Brandon, 1955). These findings served to establish that "Tingo María Fever", a disease of hitherto unknown etiology, was an acute form of pulmonary histoplasmosis.

Following these studies, a histoplasmin skin test survey of 800 Tingo María school children was carried out. More than 43% of the individuals tested gave positive reactions (Villa, Perez & Vargas, 1956), which suggested that exposure to *H. capsulatum* took place in a wider area than the confines of the oil-bird cave. To test this hypothesis, in May and Aug., 1956, soil samples were collected throughout the village of Tingo María and its surrounding areas. In addition, soil samples were obtained in the village of Iquitos (Feb., 1957) and the departments of Cusco (Aug., 1957) and Madre de Dios (July, 1958)§. These last specimens were collected to determine if ecological conditions favourable for the development of *H. capsulatum* existed in areas other than Tingo María.

## Soil Studies

The soil samples were collected predominately from avian habitats and air mailed to the Mycology Unit of the Communicable Disease Center, in waxed paper containers. Upon arrival, they were processed for the isolation of human pathogenic fungi. *H. capsulatum* and other systemic pathogens were sought by injection into mice of the supernatant from aqueous suspensions of each soil sample, and the subsequent culture of their livers and spleens. Dermatophyte isolation from some of the soil samples was carried out by baiting soil with bits of sterilized human hair. Details of both of these procedures are available in a previous publication (Ajello, Briceño-Maaz, Campins, & Moore, 1960).

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§ The soil collection in the Departments of Cusco and Madre de Dios was supported by a grant from the Medical School of Lima, Peru.

One hundred and seventeen soil samples were obtained: 47 from the Tingo María area, 24 from Iquitos, 34 from the Department of Cusco, and 12 from the department of Madre de Dios (table 1). *H. capsulatum* was recovered from 5 soil samples collected in the Tingo María area and from 1 of the Quincemil sites. *Microsporum gypseum* was isolated from 15 of the Tingo María soil samples, 3 of the Iquitos soils and from 1 of 22 Quincemil soils tested.

| *Collection Region and Habitat* | *Number* | *Pathogens Isolated* |
|---|---|---|
| DEPARTMENT OF HUÃNUCO | | |
| *Tingo María Area* | | |
| Avian habitats | 40 | *Histoplasma capsulatum*—5<br>*Microsporum gypseum*—12 |
| Yard soil | 5 | *Microsporum gypseum*—3 |
| Road side | 2 | Negative for pathogenic fungi |
| DEPARTMENT OF LORETO | | |
| *Iquitos Area* | | |
| Avian habitats | 24 | *Microsporum gypseum*—3 |
| DEPARTMENT OF CUSCO | | |
| *All from avian habitats* | | |
| Pueblo Viejo | 1 | |
| Quillabamba | 3 | |
| Quincemil | 28 | *Histoplasma capsulatum*—1<br>*Microsporum gypseum*—1 |
| San Lorenzo | 2 | |
| DEPARTMENT OF MADRE DE DIOS | | |
| *All from avian habitats* | | |
| Iberia | 3 | Negative for pathogenic fungi |
| Puerto Maldonado | 9 | ” ” ” |
| TOTAL | 117 | *H. capsulatum*—6<br>*M. gypseum*—19 |

TABLE 1.—PERUVIAN SOILS EXAMINED FOR HUMAN PATHOGENIC FUNGI.

## Discussion

The value of soil studies in the detection of endemic areas for geophilic human pathogenic fungi was well demonstrated by this study. Although only a limited number of specimens was tested, unequivocal proof of the existence of *H. capsulatum* in the Tingo María area outside of the oil-bird cave was obtained. Previously only presumptive skin test evidence for its occurrence there was available.

The study also brought to light an endemic focus for *H. capsulatum* in Quincemil, a village in the Department of Cusco. However, it failed to uncover *H. capsulatum* in the Iquitos area, where Bouroncle (1955) had reported that 337 (37%) of a group of 910 individuals tested were positive to histoplasmin.

To date, only 2 human cases of histoplasmosis have been diagnosed by culture in Peru (Celso Arellano & Galvez Brandon, 1955) although there is strong presumptive clinical evidence of an appreciable number of cases. These 2 patients were members of a group of 5 individuals who developed Tingo María Fever following a visit to the Tingo María oil bird cave.

Histoplasmin skin test surveys carried out by several investigators have uncovered several areas where sensitization to *H. capsulatum* has occurred (table 2). As yet, however, symptomatic cases of histoplasmosis have not been diagnosed in all those regions.

| *City* | *Percentage* | *Reference* |
|---|---|---|
| Callao | 24·7 | Bouroncle, 1955 |
| Callao | 2·4 | Miranda and Troncoso, 1958 |
| Ica | 1·7 | Bouroncle, 1955 |
| Iquitos | 37·0 | ” ” |
| Lima | 4·7 | ” ” |
| Tingo María | 43·0 | Villa, Perez, Vargas, 1956 |
| Ancash | 7·6 (Based on survey of 1,000 soldiers) | Miranda and Troncoso, 1958 |
| Arequipa | | ” ” ” |
| Cajamarca | | ” ” ” |
| Callao | | ” ” ” |
| Cusco | | ” ” ” |
| La Libertad | | ” ” ” |
| Lambayeque | | ” ” ” |
| Lima | | ” ” ” |
| Loreto | | ” ” ” |
| Piura | | ” ” ” |
| San Martin | | ” ” ” |
| Tumbes | | ” ” ” |

TABLE 2.—HISTOPLASMIN SKIN SENSITIVITY IN PERU

It is evident from these various types of studies that *H. capsulatum* is present in several parts of Peru and that infections occur among the population. Additional clinical and epidemiological studies need to be carried out to reveal the incidence and prevalence of histoplasmosis in this part of South America.

Soil studies have established clearly that *M. gypseum* is indigenous in the soil of many parts of Africa (Doupagne, 1959; Ajello, Manson Bahr, & Moore, 1960), Asia (Dey & Kakoti, 1955), Australia (Durie & Frey, 1955), Europe (Hejtmanek, 1957; Grin & Ozegovic, 1957; Bánhegyi, 1959), and the United States (Ajello, 1953). In Latin America, *M. gypseum* has been found in the soil of Brazil (Silva, 1960), Costa Rica (Mata & Mata, 1959), Cuba (Fuentes, Bosch, & Baudet, 1955), Ecuador (Rodriguez, 1958), Panama (Ajello, 1954), Trinidad (Ajello, Greenhall, & Moore, 1961), and Venezuela (Capretti, 1959). Its presence in Peruvian soil was to be expected. It is remarkable, however, that *Keratinomyces ajelloi* has not as yet been demonstrated in South American soils.

## ZUSAMMENFASSUNG

Bei der Untersuchung von Bodenproben aus den peruanischen Bezirken Huánuco, Loreto, Cusco und Madre de Dios wurde *Histoplasma capsulatum* aus 5 verschiedenen Proben gezüchtet, die im Dorf Tingo María und seiner Umgebung (Huánuco) gesammelt worden waren. Desgleichen fand sich der Pilz auch in einer Erdprobe aus dem Dorf Quincemil im Bezirk Cusco. Der Dermatophyt *Microsporum gypseum* fand sich in Erdproben aus den Bezirken Huánuco, Loreto und Cusco.

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# MECHANICAL VERSUS CHEMICAL KERATOLYSIS BY DERMATOPHYTES

F. Raubitschek

*Laboratory of Medical Mycology,*
*Department of Dermatology and Venereology (Felix Sagher, M.D., Director),*
*Hadassah University Hospital, Jerusalem, Israel.**

It is suggested that the concept of enzymatic keratolysis by dermatophytes stands in need of revision.

Non-keratin containing aqueous extracts from human hair and nails have been shown capable of supporting the growth of the pseudoparasitic phase of certain dermatophytes.

Destruction of such structures as hair and nails by an infecting fungus may be due to mechanical disintegration following invasion of the non-fibrillary matrix; the fungus feeding on the non-keratins without need to digest the keratin molecule.

Most textbooks of medical mycology consider that the proliferation of fungi in keratinous structures is due to the ability of the dermatophytes to digest keratin and feed on resulting breakdown products. This would imply that dermatophytes produce one or more keratolytic enzymes, a concept which mycologists have long tried to prove. Growth of dermatophytes on such keratinous substrates as hairs, horn, hooves, feathers, wool and finished woollen products, loss of weight in natural keratins incorporated in actively growing cultures (MacFayden, 1894; Roberts, 1894; Nannizzi, 1926; Williams, 1934; Rogers, Hirschmann & Humfeld, 1940; Page, 1950; Vanbreuseghem, 1952; Evolceanu & Alteras, 1960) and the appearance of amino acids in a culture medium containing growing dermatophytes and human hair, have all been considered evidence of keratin digestion (Daniels, 1953). In all these experiments the fungi grew in their saprophytic form and there was no invasion of the keratinous substrates, even when chemically altered hairs were used (Barlow & Chattaway, 1955). Invasion of hair and nails, however, has been achieved in cultures using the "pseudo-parasitic" phase of some dermatophytes (Raubitschek, 1957), but although slits, breaks and severe physical disruption were produced by the invading masses of fungal hyphae, there was no evidence of chemical breakdown of keratin (Raubitschek & Maoz, 1957).

Keratolytic enzymes have not yet been isolated from dermatophytes (Nickerson, 1947; Noval & Nickerson, 1959). It may therefore be asked whether the concept of chemical keratolysis is really necessary to explain the propagation of fungi in keratinous structures. May not the fungus find its nourishment elsewhere than in the keratin molecule?

Gradual biochemical changes in the upward moving epidermal cells finally lead to the formation of a completely keratinized structure of varying hardness. Intercellular spaces accompany the cells on their upward journey. These spaces

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contain nutrients in solution and also take up substances discarded by the cells during their gradual chemical reorganization. Some of these substances are relatively simple chemical compounds, easily extractable from keratinous tissues by hot water without changing the chemical structure of the keratin. The non-fibrillary matrix containing these "non-keratins" (Bolliger & Gross, 1952a, b, 1953, 1954, 1956) has been visualized under the electron microscope in the stratum corneum (Brody, 1960) and in wool (Rogers, 1959). Theoretically, the extractable non-keratins are capable of providing sufficient nutriment for the pseudoparasitic phase of dermatophytes, for in this form the nutritional requirements are much simpler than those of the saprophytic phase (Raubitschek, 1955).

It may therefore be suggested that, in disease fungi live on non-keratins and that the keratinous structures they invade are disrupted by sheer mechanical force. The following experiments, demonstrating that dermatophytes will grow in aqueous extracts of nails or hair, support such a theory.

## MATERIALS AND METHODS

### *Experiment 1.*

A surgically avulsed healthy-looking fingernail, microscopically free from fungous infection, was cleansed with pHiso-Hex*, sterile water, ethanol and ether, placed in 50 ml. sterile distilled water in a 125 ml. Erlenmeyer flask, and shaken for 1 week at room temperature on a Ross-Kershaw continuous shaking machine. The nail was then filtered off and the aqueous extract autoclaved. After cooling, the liquid was inoculated with 0·2 ml. of a spore suspension of *Trichophyton mentagrophytes* and replaced on the shaker at 26° C.

### *Experiment 2.*

Samples (500 mg.) of scalp hair, from female patients not suffering from fungous infections, were repeatedly washed with pHiso-Hex, rinsed thoroughly with sterile distilled water, briefly immersed in 2 changes of 96% ethanol, 1 of ether, and dried in sterile petri-dishes. The dried specimens were then placed in 50 ml. sterile distilled water in sterile Erlenmeyer flasks. These were kept in a water bath at 80° C. and shaken briskly for 2 hours. After filtering off the hair, the aqueous extracts were divided into 2 portions, each of which was placed in a 125 ml. Erlenmeyer flask and autoclaved for 15 min. at 15 atm. An equal number of flasks containing 25 ml. of sterile distilled water were also autoclaved and served as controls. All flasks were inoculated with 0·2 ml. spore suspension (Raubitschek, 1955) of either *T. mentagrophytes, T. rubrum* or *T. tonsurans*, the fungi being fresh routine isolates from patients of this department. Two extract-containing and 2 control flasks were inoculated with each organism. In each case 1 extract-containing and 1 control flask were put on a Ross-Kershaw continuous shaking machine and incubated at 26° C., whilst the other set was placed in an incubator at the same temperature.

## RESULTS

Within 2 weeks, after inoculation of the aqueous extract obtained from the fingernail, numerous pellets of *T. mentagrophytes* had grown. In experiment 2, after only 4 days, the extract-containing flasks on the shaker showed a growth of pellets whose size and number increased considerably during a further 10 days shaking. The control flasks showed no growth. The stationary extract-containing flasks showed flakes of fungal growth after 10 days, whereas there was no growth in the control flasks.

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* 3% Hexachlorophen in Lanolincholesterol and Petrolatum base by Winthrop.

## Comment

These results demonstrate that non-keratinous compounds capable of supporting the growth of some dermatophytes may be readily extracted from nails and hair. No degradation or breakdown of keratin results from the simple extraction method used in these experiments. It seems reasonable to suppose that the invasion of keratinous structures by dermatophytes is facilitated by the immediate availability of nutritive water soluble substances. Taken together with the repeated failure unequivocally to demonstrate chemical breakdown of keratin (Nickerson, 1947), this suggests that the accepted concept of enzymatic keratolysis by dermatophytes stands in need of revision.

Examples of "chemical keratolysis" previously reported are capable of an alternative interpretation. Microscopic observation of the disappearance of nail-particles in a growing dermatophyte culture does not necessarily imply that keratin has been digested. If the fungus feeds on the non-keratinous matrix of the nail and the latter becomes densely invaded by hyphae, the cohesiveness of the structure will be destroyed. The nail will then disintegrate and no longer be seen as a particle of a certain form and size. The appearance of particular amino acids in a medium in which fungus is growing in the presence of hair could be due to the metabolism of the fungus and lysis of its elements. Increased respiration after the addition of a keratinous substrate to a respiring fungus does not prove enzymatic digestion of keratin. It could equally be due to utilization of non-keratins. All the experimental evidence so far advanced to "prove" the chemical breakdown of keratin by dermatophytes can be similarly re-interpreted.

## Résumé

L'auteur suggère que soit révisée l'idée que l'on se fait actuellement de la lyse enzymatique de la kératine par les dermatophytes.

Des extraits aqueux de cheveux et d'ongles humains, exempts de kératine, permettent la croissance de la phase pseudo-parasitaire de certains dermatophytes.

La destruction de l'ensemble complexe qu'est un cheveu ou un ongle par un champignon parasite peut être due à une désintégration mécanique faisant suite à l'envahissement de la matrice non fibrillaire; la nutrition du champignon se faisant aux dépens de matériel étranger à la kératine, ne nécessite pas la lyse de la molécule de ce composé.

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# OBSERVATIONS ON THE SEASONAL VARIATIONS IN INCIDENCE OF RINGWORM IN DOGS AND CATS IN THE UNITED STATES

W. Kaplan and M. Sue Ivens

*Department of Health, Education and Welfare, Public Health Service, Communicable Disease Center, Laboratory Branch, Atlanta 22, Georgia*

The data presented in this report suggest that seasonal variations occur in the incidence of ringworm in dogs and cats in the United States. In addition, it appears that the incidence patterns, when discernible, vary with the dermatophyte involved.

The principal etiologic agents of ringworm of dogs and cats in the United States are *Microsporum canis, M. gypseum* and *Trichophyton mentagrophytes* (var. *granulare*) (Kaplan, Georg, & Ajello, 1958). Collectively these fungi are responsible for more than 99% of feline and canine dermatophytoses (Kaplan, *et al.*, 1958).

The three organisms differ in their ecology and relative importance as incitants of disease in our small-animal population. *M. canis* is responsible for about 98% of the ringworm in cats and 66% in dogs (Georg, 1960). It is also an important cause of simian ringworm and occasionally produces infections in horses. *M. canis* is essentially parasitic in habit and is transmitted from infected animals to susceptible hosts by direct or indirect contact. Although basically a zoophilic dermatophyte it is frequently implicated as a cause of ringworm in man in urban areas (Georg, 1954) and investigations frequently disclose that cats or dogs have served as vectors of infections (Georg, Hand, & Menges, 1956). Georg (1954) has found that 10-30% of ringworm of the exposed parts of the body in urban dwellers is due to this fungus.

*M. gypseum* is widely distributed in the environment as a free-living agent in soil (Gordon, Ajello, & Georg, 1952). Ajello (1953) recovered it from 37 (31·9%) of 116 soil samples collected from various sites in Georgia and Tennessee. Subsequent studies of foreign and domestic soils by several workers (Ajello, 1956; Frey & Durie, 1956) have resulted in equally frequent isolations, suggesting that animals and humans are often exposed to this agent in nature. In the United States *M. gypseum* causes close to 24% of the ringworm in dogs and 1% of the ringworm in cats (Georg, 1960). This dermatophyte is also infectious for other animals (Kaplan *et al.*, 1958). Recently Kaplan, Georg, & Fosnaugh, 1956 and Kaplan, Hopping, & Georg, 1957 reported an outbreak and several isolated cases of *M. gypseum* ringworm in horses. This geophilic fungus, however, does not appear to be an important cause of human dermatophytosis. In several surveys (Benedek & Felsher, 1944; Lewis & Hopper, 1948; Swartz, Rockwood, & Glicklich, 1949) of human ringworm *M. gypseum* has been found to be responsible for only 0·7-2·1% of the cases studied.

*T. mentagrophytes* (var. *granulare*) is an important cause of ringworm in a large variety of domesticated and wild animals (Kaplan *et al.*, 1958). In the United States it causes close to 10% of the canine and 1% of the feline dermatophytoses (Georg, 1960). *T. mentagrophytes* (var. *granulare*) is transmitted from animals to man, and human infections are encountered principally in rural areas. Georg *et al.* (1956), isolated it from about half of the cases of suppurative ringworm in humans in rural areas of central Michigan. In the past, epidemiological investigations of such human cases have usually failed to demonstrate a source of infection among animal contacts. The results of recent wild animal surveys (McKeever, Kaplan, & Ajello, 1958; McKeever, Menges, Kaplan, & Ajello, 1958; Smith, Menges, & Georg, 1957) have indicated possible sources of such infections. These studies revealed that wild animals, particularly the rodents commonly found on farm premises are frequently carriers of *T. mentagrophytes*. It is possible that rodent carriers serve as indirect sources of human and animal infections through contamination of the environment with this fungus.

Certain mycotic infections in humans and lower animals show seasonal variations in incidence. In California the incidence of human coccidioidomycosis reaches its peak during the dry, dusty, fall months and ebbs during the wet winter months (Smith, 1940; Smith, Beard, Rosenberger & Whiting, 1946). In Uruguay, Mackinnon (1949) reported that human cases of sporotrichosis occur mainly during the fall and early winter months. In the United States the incidence of bovine ringworm is at its peak during the winter months when cattle are kept in barns and during the warm months of the year there is a marked decrease in cases (Georg, 1960). The present report presents data which strongly suggest that the incidence of ringworm in dogs and cats also has seasonal variations and that the incidence patterns, when discernible, are distinct for each of the dermatophytes involved.

## Materials and Methods

The data of the present report were accumulated between Oct. 1955 and Feb. 1959 during the course of a survey of animal ringworm in the United States (Menges, 1955). This survey was conducted with the cooperation of veterinarians from all sections of the United States who submitted hairs and/or skin scrapings from animals suspected of having a fungous infection to the Mycology Laboratory, Communicable Disease Center, Chamblee, Georgia, for detailed laboratory study. The reasons for suspicion of a mycotic disease varied during the course of this study. In some cases such suspicion resulted because a human contact had a skin disorder of fungal origin in others because the appearance of the lesions was consistent with the veterinarians' concept of clinical ringworm. Still in other instances, a fungous infection was suspected because the lesions were difficult to treat.

As reported by the attending veterinarians, the skin lesions of the animals in this series varied considerably throughout the course of this study. Some animals had only a few missing or broken hairs; others had extensive heavily crusted areas of alopecia. In some cases the lesions were dry and scaly; in others they were moist and suppurative. Frequently the clinicians stated that the lesions were circular, scaly patches of alopecia. No attempt was made to restrict the submission of specimens to certain types of lesions.

In addition to Wood's light and direct microscopic examinations, all specimens were cultured on a selective isolation medium (Georg, Ajello & Papageorge, 1954) containing cycloheximide*, penicillin, and streptomycin. Each sample was inoculated into a minimum of 3 tubes of medium and incubated at room temperature (25°C.) for 1 month before being discarded.

## RESULTS

Clinical materials from 3,914 dogs and 1,232 cats suspected of having ringworm were received. These were submitted by veterinarians from 35 states in all sections of the country (tables 1 and 2). Seven hundred and seventy-five (19·8%) of the dogs and 379 (30·8%) of the cats were found to be infected with either *M. canis, M. gypseum,* or *T. mentagrophytes* (var. *granulare*). The infected animals had resided in 29 different states.

### *M. canis Infections in Dogs*

Of the 3,914 dogs, 501 (12·8%) were found to be infected with *M. canis* (table 1) and this condition was diagnosed during every month of the study period. However, the infection rates among the animals in this series varied from month to month. As shown in table 1 and fig. 1, the highest rates were found from Oct. to Feb. The average rate during each month being 16·4%, 17·4%, 19·9%, 16·3% and 15·5% respectively. During 1957 and 1958 the rates declined sharply in March, followed by a small secondary rise in April. In 1956 the secondary increase occurred in March. The incidence of this infection in dogs was low from March to Sept. The average infection rates during these months were 9·0%, 11·5%, 7·0%, 6·2%, 8·7%, 9·3% and 8·9%.

There was a progressive decline each year in the number of dogs infected with *M. canis*. In 1956 the overall rate was 14·0%, in 1957 it was 13·1% and in 1958 it dropped to 9·3%. This trend is readily apparent when the low incidence months of each year are compared (fig. 1). However, the rates during the high incidence months remained constant.

### *M. gypseum Infections in Dogs*

Of the 3,914 dogs investigated 191 (4·9%) were found to have ringworm caused by *M. gypseum* (table 1). However, the infection rates fluctuated markedly from month to month. As shown in table 1, fig. 1 the highest numbers of *M. gypseum* infections in dogs occurred from July to Nov. The average infection rate for each month was 9·0%, 9·3%, 9·8%, 9·7% and 8·7% respectively. During the years 1956, 1957, and 1958 the rates dropped sharply in Dec. and remained at a low level until the following July, at which time a sharp rise occurred. In 1955 there was a drop in Dec.; however, the marked downturn occurred in the following Jan. The low months for the occurrence of this infection were from Dec. to June. The average rate for each month was 2·8%, 1·1%, 1·0%, 0·0%, 0·4%, and 1·8%.

The annual rate of *M. gypseum* infections remained relatively constant

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*Acti-dione, manufactured by the Upjohn Co., Kalamazoo, Michigan. Trade names are used for identification purposes only and mention of them does not constitute an endorsement by the Public Health Service.

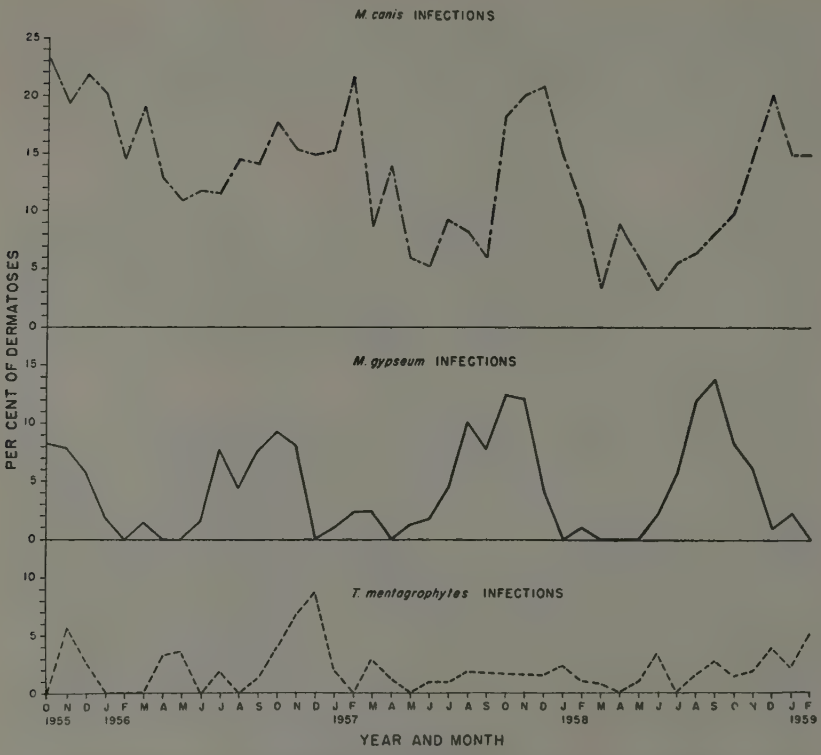

Figure 1.—Isolation of Dermatophytes from Dogs in the United States, Oct. 1955-Feb. 1959.

during each year of this study. In 1956 the overall rate was 4·3%, in 1957, 5·5% and in 1958, 4·7%.

Since the natural habitat of *M. gypseum* is soil, it was felt that climatic conditions in a given geographic area probably influenced the occurrence of infections with this agent. Accordingly, it was decided to compare the incidence of this infection in dogs in northern and southern regions of the United States. The results of such a comparison are noted in table 3 and fig. 3. The northern region comprised the New England States, New York, New Jersey, Pennsylvania Ohio, Indiana, Michigan, Illinois, Wisconsin, Minnesota, Iowa, Missouri, Kansas and Nebraska. The southern region encompassed Virginia, North Carolina, South Carolina, Kentucky, Tennessee, Georgia, Florida, Alabama, Mississippi, Louisiana, Arkansas, and Texas. As shown in table 3, 2,145 dogs from the northern region were investigated and 74 (3·45%) were found to be infected with *M. gypseum*. Of the 1,387 southern dogs cultured, 111 (8·0%) had ringworm caused by this agent. The peak months for the occurrence of this

| Year | Month | No. dogs cultured | No. *M. canis* cases | % *M. canis* | No. *M. gypseum* cases | % *M. gypseum* | No. *T. mentagrophytes* cases | % *T. mentagrophytes* | Total ringworm cases | % ringworm |
|---|---|---|---|---|---|---|---|---|---|---|
| 1955 | Oct. | 73 | 17 | 23·2 | 6 | 8·2 | 0 | 0 | 23 | 31·5 |
| | Nov. | 88 | 17 | 19·3 | 7 | 7·9 | 5 | 5·7 | 29 | 32·9 |
| | Dec. | 69 | 15 | 21·7 | 4 | 5·8 | 2 | 2·9 | 21 | 30·4 |
| 1956 | Jan. | 54 | 11 | 20·1 | 1 | 1·8 | 0 | 0 | 12 | 22·2 |
| | Feb. | 61 | 9 | 14·7 | 0 | 0 | 0 | 0 | 9 | 14·7 |
| | March | 69 | 13 | 18·8 | 1 | 1·4 | 0 | 0 | 14 | 20·3 |
| | April | 63 | 8 | 12·7 | 0 | 0 | 2 | 3·2 | 10 | 15·9 |
| | May | 65 | 6 | 10·9 | 0 | 0 | 2 | 3·6 | 8 | 12·3 |
| | June | 69 | 8 | 11·6 | 1 | 1·4 | 0 | 0 | 9 | 13·0 |
| | July | 104 | 12 | 11·5 | 8 | 7·6 | 2 | 1·9 | 22 | 21·1 |
| | Aug. | 90 | 13 | 14·4 | 4 | 4·4 | 0 | 0 | 17 | 18·9 |
| | Sept. | 80 | 11 | 13·7 | 6 | 7·5 | 1 | 1·2 | 18 | 22·5 |
| | Oct. | 107 | 19 | 17·7 | 10 | 9·3 | 4 | 3·7 | 33 | 30·8 |
| | Nov. | 135 | 21 | 15·5 | 11 | 8·1 | 9 | 6·6 | 41 | 30·4 |
| | Dec. | 80 | 12 | 15·0 | 0 | 0 | 7 | 8·7 | 19 | 23·7 |
| 1957 | Jan. | 98 | 17 | 15·3 | 1 | 1·0 | 2 | 2·0 | 20 | 20·4 |
| | Feb. | 87 | 19 | 21·8 | 2 | 2·3 | 0 | 0 | 21 | 24·1 |
| | March | 102 | 9 | 8·8 | 3 | 2·4 | 3 | 2·9 | 15 | 14·7 |
| | April | 79 | 11 | 13·9 | 0 | 0 | 1 | 1·3 | 12 | 15·2 |
| | May | 83 | 5 | 6·0 | 1 | 1·2 | 0 | 0 | 6 | 7·2 |
| | June | 114 | 6 | 5·2 | 2 | 1·7 | 1 | 0·9 | 9 | 7·9 |
| | July | 131 | 12 | 9·2 | 6 | 4·6 | 1 | 0·8 | 19 | 14·5 |
| | Aug. | 108 | 9 | 8·3 | 11 | 10·1 | 2 | 1·8 | 22 | 20·4 |
| | Sept. | 116 | 7 | 6·0 | 9 | 7·7 | 2 | 1·7 | 18 | 15·5 |
| | Oct. | 121 | 22 | 18·2 | 15 | 12·4 | 2 | 1·6 | 39 | 32·2 |
| | Nov. | 123 | 25 | 20·3 | 15 | 12·1 | 2 | 1·6 | 42 | 34·1 |
| | Dec. | 124 | 27 | 21·8 | 5 | 4·0 | 2 | 1·6 | 34 | 27·4 |
| 1958 | Jan. | 122 | 18 | 14·7 | 0 | 0 | 3 | 2·4 | 21 | 17·2 |
| | Feb. | 95 | 10 | 10·5 | 1 | 1·0 | 1 | 1·0 | 12 | 12·6 |
| | March | 118 | 4 | 3·4 | 0 | 0 | 1 | 0·8 | 5 | 4·2 |
| | April | 76 | 6 | 7·9 | 0 | 0 | 0 | 0 | 6 | 7·9 |
| | May | 95 | 6 | 6·3 | 0 | 0 | 1 | 1·0 | 7 | 7·4 |
| | June | 91 | 3 | 3·3 | 2 | 2·2 | 3 | 3·3 | 8 | 8·8 |
| | July | 108 | 6 | 5·5 | 8 | 7·4 | 0 | 0 | 14 | 12·9 |
| | Aug. | 125 | 8 | 6·4 | 15 | 12·0 | 2 | 1·6 | 25 | 20·0 |
| | Sept. | 109 | 9 | 8·2 | 15 | 13·8 | 3 | 2·7 | 27 | 24·8 |
| | Oct. | 133 | 13 | 9·8 | 11 | 8·3 | 2 | 1·5 | 26 | 19·5 |
| | Nov. | 113 | 17 | 15·0 | 7 | 6·1 | 7 | 1·9 | 31 | 27·4 |
| | Dec. | 89 | 18 | 20·2 | 1 | 1·1 | 3 | 3·4 | 22 | 24·7 |
| 1959 | Jan. | 87 | 13 | 15·0 | 2 | 2·3 | 2 | 2·3 | 17 | 19·5 |
| | Feb. | 60 | 9 | 15·0 | 0 | 0 | 3 | 5·0 | 12 | 20·0 |
| TOTAL | | 3,914 | 501 | 12·8 | 191 | 4·9 | 83 | 2·1 | 775 | 19·8 |

TABLE 1. ISOLATION OF DERMATOPHYTES FROM DOGS IN THE UNITED STATES OCTOBER 1955-FEBRUARY 1959

infection in the northern dogs were Aug., Sept., Oct., and Nov. The average infection rate for each month was 9·5 %, 8·3 %, 6·0 %, and 6·3 % respectively. The rate dropped sharply to 3·3 % in December. During the months from Jan. to June the infection was virtually non-existent in the northern canines. In July, the average infection rate rose to 3·0 %. In the southern dogs, the peak months for the occurrence of this disorder were from July to Nov. The average infection rate for each month was 11·9%, 11·1%, 14·3%, 15·9%, and 15·2%. The rate dropped sharply to 3·0% in December. During Jan. and Feb. the occurrence of this disease was at a low level with infection rates of 0·8%, and 1·7% respectively. In March a small secondary rise in incidence occurred and

| Year | Month | No. cats cultured | No. *M. canis* cases | % *M. canis* | No. *M. gypseum* cases | % *M. gypseum* | No. *T. mentagrophytes* cases | % *T. mentagrophytes* | Total ringworm cases | % ringworm |
|---|---|---|---|---|---|---|---|---|---|---|
| 1955 | Oct. | 27 | 9 | 33·3 | 2 | 8·0 | — | — | 11 | 40·7 |
| | Nov. | 17 | 9 | 53·9 | 1 | 5·9 | 1 | 5·8 | 11 | 64·7 |
| | Dec. | 20 | 7 | 35·0 | — | — | — | — | 7 | 35·0 |
| 1956 | Jan. | 27 | 13 | 48·1 | — | — | — | — | 13 | 48·1 |
| | Feb. | 22 | 10 | 45·4 | — | — | 1 | 4·5 | 11 | 50·0 |
| | March | 15 | 5 | 33·3 | — | — | — | — | 5 | 33·3 |
| | April | 25 | 9 | 36·0 | — | — | — | — | 9 | 36·0 |
| | May | 17 | 3 | 17·6 | — | — | — | — | 3 | 17·6 |
| | June | 20 | 7 | 35·0 | — | — | — | — | 7 | 35·0 |
| | July | 28 | 10 | 35·7 | 1 | 3·5 | — | — | 11 | 39·3 |
| | Aug. | 23 | 10 | 43·5 | — | — | — | — | 10 | 43·5 |
| | Sept. | 18 | 11 | 61·1 | — | — | — | — | 11 | 61·1 |
| | Oct. | 41 | 13 | 31·7 | — | — | — | — | 13 | 31·7 |
| | Nov. | 41 | 15 | 36·6 | — | — | — | — | 15 | 36·6 |
| | Dec. | 28 | 11 | 39·3 | 1 | 3·5 | — | — | 12 | 42·8 |
| 1957 | Jan. | 30 | 10 | 33·3 | — | — | — | — | 10 | 33·3 |
| | Feb. | 37 | 12 | 32·4 | — | — | 1 | 2·7 | 13 | 35·1 |
| | March | 27 | 9 | 33·3 | — | — | — | — | 9 | 33·3 |
| | April | 34 | 10 | 29·4 | — | — | — | — | 10 | 29·4 |
| | May | 24 | 7 | 29·2 | — | — | — | — | 7 | 29·2 |
| | June | 32 | 5 | 15·6 | — | — | — | — | 5 | 15·6 |
| | July | 37 | 11 | 29·7 | — | — | — | — | 11 | 29·7 |
| | Aug. | 26 | 8 | 30·8 | — | — | 1 | 3·8 | 9 | 34·6 |
| | Sept. | 29 | 7 | 24·1 | — | — | — | — | 7 | 24·1 |
| | Oct. | 36 | 13 | 36·1 | — | — | — | — | 13 | 36·1 |
| | Nov. | 26 | 8 | 30·8 | — | — | — | — | 8 | 30·8 |
| | Dec. | 31 | 14 | 45·2 | — | — | 1 | 3·2 | 15 | 48·4 |
| 1958 | Jan. | 34 | 7 | 20·6 | — | — | 1 | 2·9 | 8 | 23·5 |
| | Feb. | 33 | 4 | 12·1 | — | — | — | — | 4 | 12·1 |
| | March | 32 | 6 | 18·7 | — | — | — | — | 6 | 18·7 |
| | April | 27 | 6 | 22·2 | — | — | 1 | 3·7 | 7 | 25·9 |
| | May | 30 | 9 | 30·0 | — | — | — | — | 9 | 30·0 |
| | June | 33 | 6 | 18·2 | — | — | 1 | 3·0 | 7 | 21·2 |
| | July | 37 | 7 | 18·9 | — | — | — | — | 7 | 18·9 |
| | Aug. | 30 | 8 | 26·6 | — | — | — | — | 8 | 26·6 |
| | Sept. | 28 | 11 | 39·2 | — | — | — | — | 11 | 39·2 |
| | Oct. | 49 | 6 | 12·2 | — | — | — | — | 6 | 12·2 |
| | Nov. | 49 | 6 | 12·2 | — | — | 1 | 2·0 | 7 | 14·3 |
| | Dec. | 26 | 6 | 23·1 | 1 | 3·8 | — | — | 7 | 26·9 |
| 1959 | Jan. | 48 | 16 | 33·3 | — | — | 1 | 2·1 | 17 | 35·4 |
| | Feb. | 38 | 9 | 23·7 | — | — | — | — | 9 | 23·7 |
| TOTAL | | 1,232 | 363 | 29·5 | 6 | 0·5 | 10 | 0·8 | 379 | 30·8 |

TABLE 2. ISOLATION OF DERMATOPHYTES FROM CATS IN THE UNITED STATES, OCTOBER 1955-FEBRUARY 1959

| Year | Month | NORTHERN REGION | | | SOUTHERN REGION | | |
|---|---|---|---|---|---|---|---|
| | | No. dogs cultured | No. *M. gypseum* cases | % *M. gypseum* | No. dogs cultured | No. *M. gypseum* cases | % *M. gypseum* |
| 1955 | Oct. | 46 | 3 | 6·5 | 22 | 3 | 13·6 |
| | Nov. | 65 | 3 | 4·6 | 15 | 3 | 20·0 |
| | Dec. | 48 | 4 | 8·3 | 11 | 0 | 0 |
| 1956 | Jan. | 31 | 0 | 0 | 16 | 0 | 0 |
| | Feb. | 28 | 0 | 0 | 28 | 0 | 0 |
| | March | 43 | 0 | 0 | 24 | 1 | 4·2 |
| | April | 48 | 0 | 0 | 13 | 0 | 0 |
| | May | 39 | 0 | 0 | 10 | 0 | 0 |
| | June | 39 | 0 | 0 | 27 | 1 | 3·7 |
| | July | 57 | 2 | 3·5 | 41 | 6 | 14·6 |
| | Aug. | 47 | 4 | 8·5 | 38 | 0 | 0 |
| | Sept. | 46 | 3 | 6·5 | 29 | 3 | 10·3 |
| | Oct. | 53 | 3 | 5·7 | 48 | 6 | 12·5 |
| | Nov. | 79 | 5 | 6·3 | 52 | 6 | 11·5 |
| | Dec. | 42 | 0 | 0 | 30 | 0 | 0 |
| 1957 | Jan. | 46 | 0 | 0 | 40 | 1 | 2·5 |
| | Feb. | 39 | 0 | 0 | 41 | 2 | 4·9 |
| | March | 46 | 0 | 0 | 52 | 3 | 5·8 |
| | April | 39 | 0 | 0 | 27 | 0 | 0 |
| | May | 31 | 0 | 0 | 34 | 1 | 2·9 |
| | June | 44 | 0 | 0 | 56 | 2 | 3·6 |
| | July | 59 | 0 | 0 | 59 | 6 | 10·2 |
| | Aug. | 42 | 3 | 7·1 | 52 | 8 | 15·4 |
| | Sept. | 51 | 1 | 1·9 | 56 | 8 | 14·3 |
| | Oct. | 59 | 4 | 6·8 | 48 | 11 | 22·9 |
| | Nov. | 79 | 8 | 10·1 | 37 | 7 | 18·9 |
| | Dec. | 74 | 3 | 4·1 | 38 | 2 | 5·3 |
| 1958 | Jan. | 79 | 0 | 0 | 36 | 0 | 0 |
| | Feb. | 57 | 0 | 0 | 33 | 0 | 0 |
| | March | 70 | 0 | 0 | 24 | 0 | 0 |
| | April | 41 | 0 | 0 | 27 | 0 | 0 |
| | May | 59 | 0 | 0 | 31 | 0 | 0 |
| | June | 45 | 0 | 0 | 35 | 2 | 5·7 |
| | July | 52 | 3 | 5·8 | 43 | 5 | 11·6 |
| | Aug. | 80 | 9 | 11·2 | 36 | 6 | 16·7 |
| | Sept. | 60 | 9 | 15·0 | 34 | 6 | 17·6 |
| | Oct. | 75 | 4 | 5·3 | 46 | 6 | 13·0 |
| | Nov. | 65 | 2 | 3·1 | 34 | 5 | 14·7 |
| | Dec. | 51 | 0 | 0 | 20 | 1 | 5·0 |
| 1959 | Jan. | 54 | 1 | 1·8 | 26 | 0 | 0 |
| | Feb. | 37 | 0 | 0 | 18 | 0 | 0 |
| TOTAL | | 2,145 | 74 | 3·45 | 1,387 | 111 | 8·0 |

TABLE 3. ISOLATION OF *Microsporum gypseum* FROM DOGS IN NORTHERN AND SOUTHERN REGIONS OF THE UNITED STATES, OCTOBER 1955-FEBRUARY 1959

the rate of infection was 4·0%. In April and May the average monthly rate fell to 0·0% and 1·3% respectively and in June it rose to 4·2%.

### *T. mentagrophytes* (*var. granulare*) *Infections in Dogs*

As shown in table 1, of the 3,914 dogs cultured, 83 (2·1%) were found to have ringworm caused by *T. mentagrophytes* (var. *granulare*). Although cases of this disorder in dogs were diagnosed during all months of the year the 2 peak months were Nov., with an average rate of 5·0% and Dec. with a rate of 3·9%. The average rates for the other months varied from 1·0% to 2·0%.

The annual rates of *T. mentagrophytes* infections in the dogs remained relatively constant during the period of this study. For 1956, the overall rate was 2·8%, for 1957, 1·4%, and for 1958, 2·0%.

### *M. canis Infections in Cats*

Of the 1,232 cats cultured, 363 (29·5%) were found to have ringworm due to *M. canis* (table 2) and this infection was diagnosed in cats during every month of the year. However, the rates varied little from month to month, the average infection rate for each month being Jan.-33·1%, Feb.-26·9%, March-27·0%, April-29·1%, May-26·8%, June-21·2%, July-27·5%, Aug.-33·0%, Sept-38·7%, Oct.-26·8%, Nov.-28·6%, Dec.-36·2%.

During the study period there was a progressive decline each year in *M. canis* ringworm in cats. In 1956, the overall rate was 38·4%, in 1957 it was 30·9% and in 1958 it was 20·1%. This trend is illustrated in fig. 2.

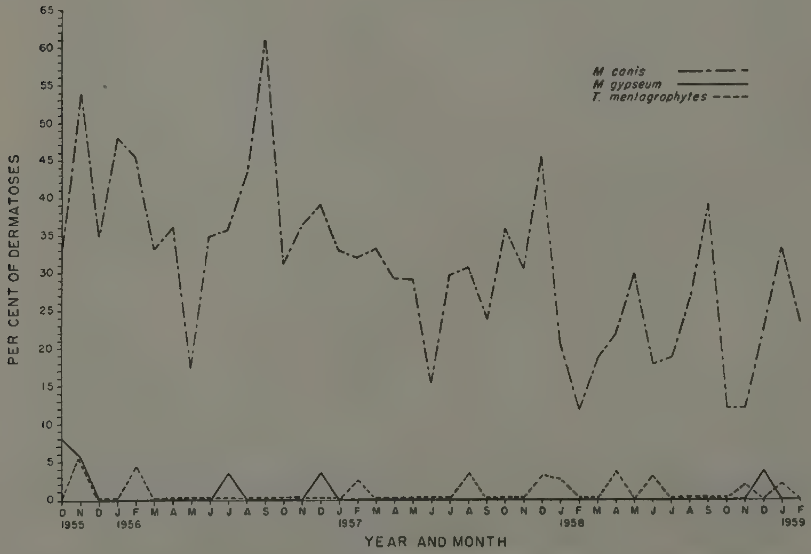

Figure 2.—Isolation of Dermatophytes from Cats in the United States, Oct. 1955-Feb. 1959.

### *M. gypseum and T. mentagrophytes (var. granulare) Infections in Cats.*

Of the 1,232 cats cultured, 6 (0·5%) were found to be infected with *M. gypseum* and 10 (0·8%) with *T. mentagrophytes* (var. *granulare*) (table 2). Two of the *M. gypseum* infections occurred in Oct., 1 in Nov., 1 in July, and 2 in Dec. These months fall into the period when *M. gypseum* infections in dogs were at

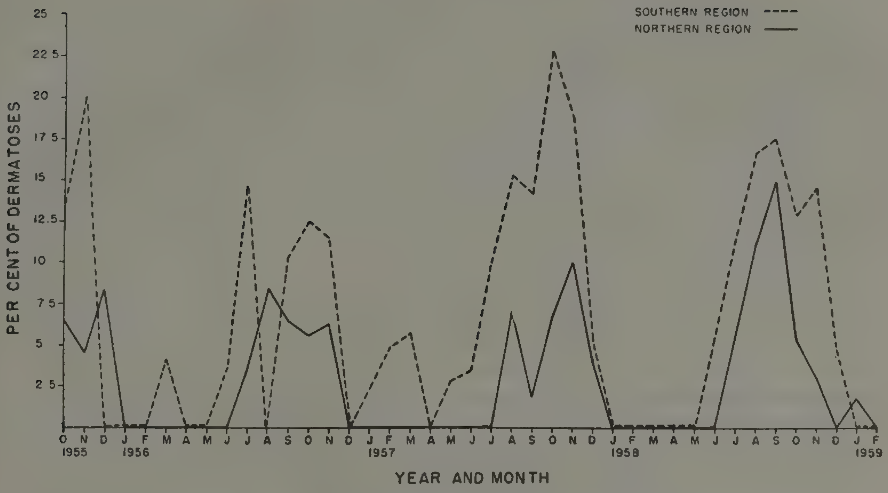

Figure 3.—Isolation of *Microsporum gypseum* from Dogs in Northern and Southern Regions of the United States, Oct. 1955-Feb. 1959.

their peak. Of the 10 cases of *T. mentagrophytes* ringworm, 2 occurred in Jan., 2 in Feb., 1 in April, 1 in June, 1 in Aug., 2 in Nov., and 1 in Dec. This relatively rare infection in cats was encountered during all seasons of the year with the majority of cases occurring during the late fall and winter.

## Discussion

The data presented in this report suggest that seasonal variations occur in the incidence of ringworm in dogs and cats in the United States, Furthermore, it appears that the patterns of incidence, when discernible, vary according to the dermatophyte involved. Thus, it is imperative to designate the causal agent when reference is made to seasonal occurrences of canine or feline dermatophytoses.

*M. gypseum* is an important cause of ringworm in dogs and infections appear to occur mainly during the summer and fall. In the winter and spring cases are virtually non-existent in the North whereas in the South a few cases do occur during this period. In addition, the overall infection rate is twice as high in the South as in the North.

Why *M. gypseum* ringworm in dogs shows such a well defined seasonal incidence cannot be adequately answered at this time. The prevalence of this dermatophyte in soil leads to the conclusion that soil must be the main source of canine infections. It would follow that exposure to this reservoir is required for infection to develop. Thus, the absence of *M. gypseum* ringworm in dogs residing in the North during the winter and early spring may be explained by the fact that a combination of cold weather, blankets of ice and snow, and the general frozen state of the soil, minimize the possibility of close contact with the reservoir of infection. However, this explanation does not account fully for the marked diminution of cases in the South during the winter and spring. In the lower tier of states the weather is mild and the ground is not frozen during these seasons of the year. However, winter and spring are usually wet seasons in the South and it is possible that the prolonged rains and excessive moisture in the soil are not conducive to close contact with this reservoir of infection. It is also possible that, both in the North and in the South, physical and microbiological conditions of the soil during the summer and autumn months are favourable for the multiplication and concentration of the fungus in the surface layer. Quantitative as well as qualitative analyses of soil samples collected at all seasons of the year would be required to confirm this hypothesis. Such explanations however, overlook the possibility that environmental conditions during the summer and fall may in some way render the canine skin more susceptible to infection with *M. gypseum*.

*M. gypseum* is a minor cause of ringworm in cats. Consequently, the number of cases diagnosed during this study was insufficient to detect a pattern. However, it is of interest to note that all the feline infections also occurred during the summer and fall months.

Ringworm in dogs due to *M. canis* also appears to have a well defined seasonal incidence. However, the pattern differs from the one noted for *M. gypseum*. In contrast to the latter type of ringworm, the peak seasons for the occurrence of *M. canis* infections in dogs are fall and winter. During the spring and summer months infections by this agent are apparently at their ebb. It is difficult to account for this seasonal pattern. *M. canis* infections spread from one host to another through direct or indirect contact. It would appear that during the fall and winter months factors such as increased opportunity for contact with the agent and reduction in cutaneous resistance to infection may be in operation.

In contrast to the clear cut pattern of incidence of *M. canis* infections in dogs, the seasonal incidence of this form of ringworm in cats is poorly defined. The data indicate that the peak months for the occurrence of this feline disease are Aug. and Sept. and Dec. and Jan. The low incidence months are May and June. However, *M. canis* infections are common in cats during the other months of the year. It appears that the factors which are conducive to the spread of this disease among cats are in operation throughout the year.

It is of interest to note that during this 41-month study there was a progressive decline each year in the incidence of ringworm due to *M. canis* in both the cats and dogs. In 1956, the overall infection rate in the cats was 38·4% and in the dogs, 14·6%. In 1957 the rates were 30·9% and 13·1% respectively, and in 1958 they dropped to 20·1% and 9·3% respectively. Statistical analysis of these figures shows that the progressive decrease is significant at the 95% level. However, it must be considered that this trend could merely reflect an unknown bias in selection of cases for mycological study. If such a bias were not in operation, then one can speculate that there is a cycle of *M. canis* ringworm among dogs and cats, with the year 1958 approaching the cyclical trough. Unfortunately the duration of the study was too short to allow definition and characterization of such a cycle, if indeed, one does exist. The confirmation of the occurrence of such a cycle and its accurate description would be of practical importance, since it would allow forecasting of morbidity rates.

The study data indicate that *T. mentagrophytes* (var. *granulare*) is an important cause of ringworm in dogs. This type of ringworm apparently occurs throughout the year with Nov. and Dec. the peak incidence months. Additional studies are required, however, to permit a definition of a seasonal pattern of occurrence of *T. mentagrophytes* ringworm among dogs. This form of ringworm in cats is rare.

## ZUSAMMENFASSUNG

Die in diesen Bericht mitgeteilten Daten deuten darauf hin, daβ in den U.S.A. der Befall von Hunden und Katzen mit Hautflechten jahreszeitlichen Schwankungen unterliegt. Ferner scheint es, daβ Art und Weise des Befalls bei den einzelnen Erregern verschieden sind.

*M. gypseum* ist eine wichtige Ursache für Hautflechten beim Hund und für etwa 24% der Hunde-Dermatophytien verantwortlich. Die mitgeteilten Befunde zeigen, daβ solche Infektionen hauptsächlich im Sommer und Herbst auftreten und, in Abhängigkeit vom jeweiligen Gebiet, im Winter wie Frühling entweder vermindert oder gar nicht vorkommen. Der gleiche Pilz verursacht nur etwa 1% der Dermatophytien bei Katzen. Die wenigen beoblachteten Fälle sind auf Sommer und Herbst beschränkt. Mögliche Erklärungen für diese interessante jahreszeitliche Verteilung der *M. gypseum*-Vorkommen werden diskutiert.

In den Vereinigten Staaten ist *M. canis* für etwa 66% der Dermatophytien beim Hund und für 98% der Fälle bei der Katze verantwortlich. Der Höhepunkt des Befalls liegt beim Hund in den Herbst- und Wintermonaten, während einschlägige Infektionen in den übrigen Jahreszeiten selten sind. Demgegenüber sind *M. canis*—Infektionen bei Katzen das ganze Jahr über häufig.

*T. mentagrophytes* var. *granulosum* ist eine alltägliche Ursache von Hautflechten beim Hund und verursacht etwa 10% der Dermatophytien, Im Gegensatz dazu ist es relativ selten Erreger von Katzen-Dermatophytie. Der Höhepunkt des Befalls liegt beim Hund im November und Dezember. Weitere Studien sind jedoch nötig, um die jahreszeitliche Verteilung von *T. mentagrophytes*-Infektionen bei beiden Tierarten festzustellen.

Haupterreger von Flechten bei Hund und Katze sind in den U.S.A. *Microsporum canis, M. gypseum* und *Trichophyton mentagrophytes,* (var. *granulare*) (Kaplan, Georg & Ajello, 1958). Diese Pilze sind für insgesamt mehr als 99% der Hautmykosen beider Tierarten verantwortlich (Kaplan et al., 1958).

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# GRISEOFULVIN TREATMENT IN EXPERIMENTAL *MICROSPORUM CANIS* INFECTION IN THE CAT

J. G. O'Sullivan

*Department of Veterinary Pathology,*
*University of Glasgow*

Orally administered griseofulvin caused *Microsporum canis* infection to disappear from within the hair follicles in artificially infected cats. At the same time, clinical evidence of infection also disappeared although fluorescence persisted. Removal of the distal part of affected hairs in conjunction with the application of an anti-fungal preparation reduced the period of positive fluoresence and of the recovery of the fungus in culture.

The lesions caused by *Microsporum canis* in cats are generally minimal (La Touche, 1953), in marked contrast to those produced by *Trichophyton verrucosum* in cattle. The course of the disease is often protracted. Rebell, Timmons, Lamb, Hicks, Groves & Coalson (1956) found positively fluorescing hairs 6 months after initial infection and clinical signs lasted from 1 to 6 months. Reiss, Caroline & Leonard (1955) found that the mean duration in 6 cats of clinical signs was 23 days, of positive fluorescence 121 days and of positive culture 138 days. They also noted a bluish fluorescence taking the place of the normal green. Kaplan & Ajello (1959, 1960) used griseofulvin to shorten the period of clinical and mycological involvement. In addition to the administration of griseofulvin *by mouth* they clipped the fluorescent tips of the hairs and bathed a number of the cats in Captan* solution. They concluded that although griseofulvin demonstrated a marked capacity to bring about rapid clinical recovery it was not as effective in producing a mycological recovery.

The routine of oral dosing, clipping and bathing was used by Donovan & Bohl (1960) and they found that most of their cats were clinically negative after 2 weeks although 2 animals took 8 weeks to be clinically normal.

Microsporosis in cats is refractory in nature and an important zoonosis. It therefore seemed that an investigation, designed to add further information on the course of the disease and on the effect of treatment, would be useful.

## MATERIALS AND METHODS

Seven kittens, 5 weeks old, which were free of *M. canis* infection were inoculated with a 22-day culture of *M. canis* grown on 4% malt extract agar, the strain coming from a naturally occurring case of microsporosis in a cat. A small amount of culture and agar was rubbed into a 15 cm. × 2·5 cm. area of clipped skin on 1 side of the backbone and an Elizabethan collar was put on the cat to prevent it licking off the inoculum. The cats were housed in separate cages. Starting from the day of inoculation a biopsy was taken from each cat at regular intervals using the apparatus described by

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*N—trichloromethyl mercapto—4—cyclohexene 1, 2—dicarboximide.

Evans, Nisbet & Ross (1957). This is a hollow bit, inside diameter 5 mm. operated by an electric drill turning at 2,000 r.p.m. When infection was established (in 12 days) the cats were divided into 2 groups. Nos. 1-4 were left untreated while Nos. 5, 6 and 7 received 60 mg./kg. griseofulvin in tablet form daily. These cats did not receive any topical treatment.

On the 54th day after inoculation the 4 cats (Nos. 1, 2, 3 and 4) which had until then been untreated were made the subject of a further small trial. Nos. 1, 2 and 3 were orally dosed with 60 mg./kg. griseofulvin daily for 5 days and griseofulvin cream (10% griseofulvin in liquid paraffin) was rubbed on the affected area daily for 23 days. The hair was clipped at the start of treatment and on the 9th and 16th days. Cat No. 4 was left untreated.

## Results

### *Normal course of infection from day of inoculation until 52nd day*

In the untreated animals skin thickening was detected by palpation between the 18th and 20th days after inoculation and persisted until the 52nd day, a mean duration of 33 days (fig. 1). The cats showed areas of fluorescent hairs extending up to 15 × 7-8 cm. Typical fluorescence was noted on the 12th day (in cat No. 1 it was evident on the 9th day) and persisted until the 52nd day. A bluish fluorescence was seen between the 20th and 31st day and, while the extra-follicular part of the affected hairs showed this altered coloration, the intrafollicular part of the plucked hair fluoresced typically greenish.

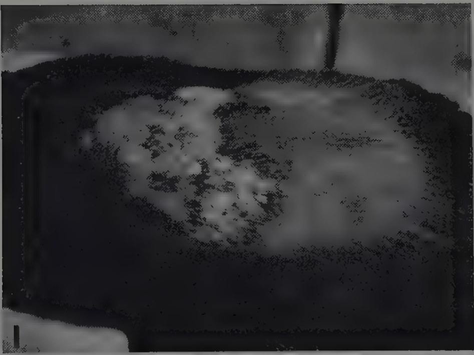
### *Treatment experiment I.*

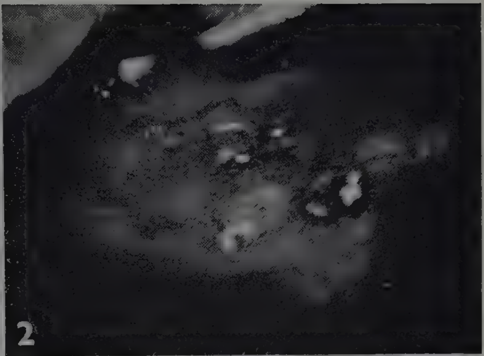
When fluorescence was demonstrable in cats Nos. 5, 6 and 7 they began to receive 40 daily doses of griseofulvin at the level of 60 mg./kg. Clinical lesions became evident between the 4th and 8th day despite treatment. However, cats Nos. 5 and 7 were clinically normal 5-9 days later at the end of 13 days treatment (table 1) and cat No. 6 after 15 treatments, although this animal's coat remained very thin. The inoculated sites in all 3 animals remained clinically normal until the end of the experiment (fig. 2).

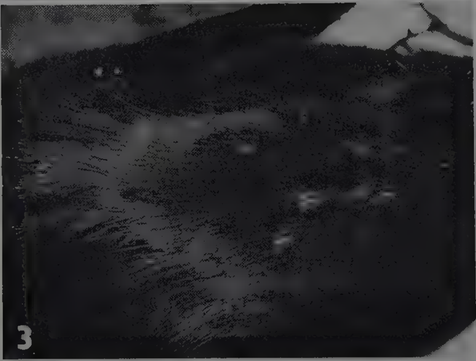
Positive fluorescence persisted in the 3 treated cats during the course of

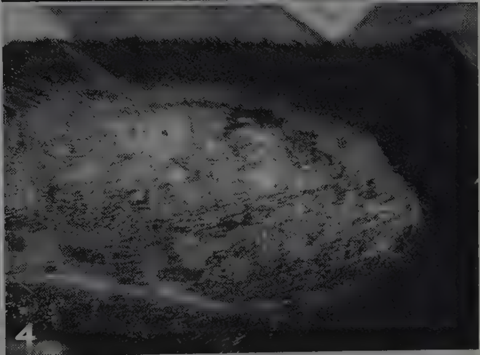
| *CAT* | *Duration in days of:—* | | |
|---|---|---|---|
| | *Clinical signs* | *Positive Fluorescence* | |
| 1 | 33 | 43 | Untreated |
| 2 | 32 | 40 | |
| 3 | 34 | ,, | |
| 4 | 34 | ,, | |
| 5 | 13 | ,, | Treated |
| 6 | 15 | ,, | |
| 7 | 13 | ,, | |

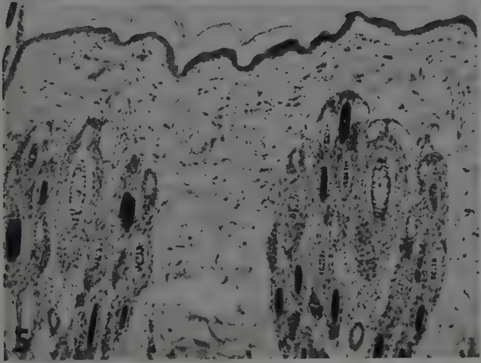
Table 1.—Effect of Orally Administered Griseofulvin (60 mg./kg. Daily 12th to 54th Day After Inoculation) to Cats Infected With *M. canis*.

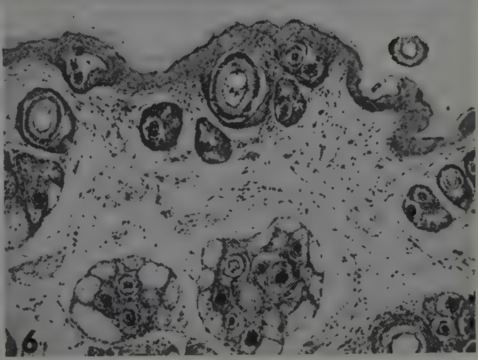








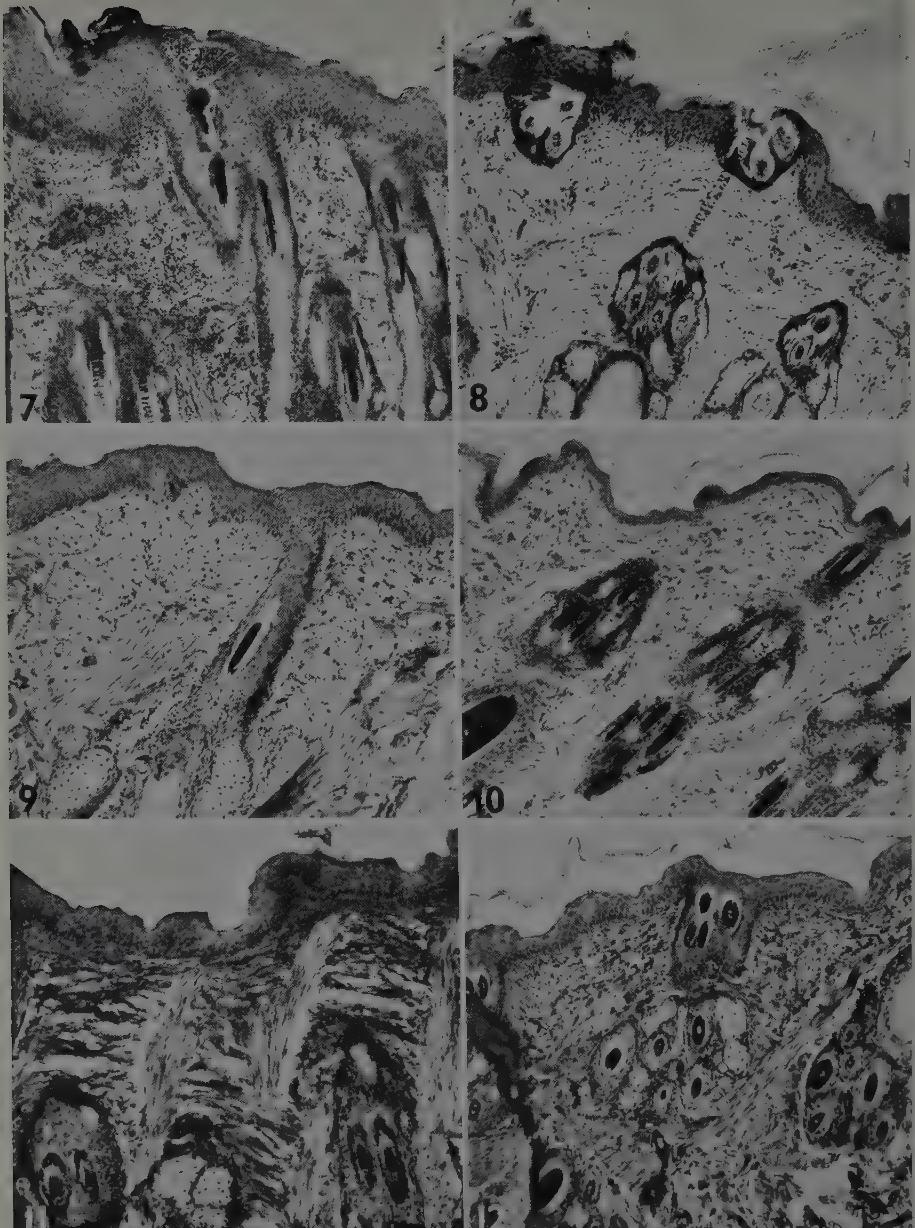




Figures 1-4—Inoculated sites on backs of cats. fig. 1) Untreated, 52 days after inoculation to show crusting of inoculated area. fig. 2) Treated, 52 days after inoculation; 40 oral treatments; no topical treatment or hair clipping. Skin normal apart from biopsy scars. fig. 3) Treated orally for 5 days, topically for 12 days. fig. 4) Untreated at same time as fig. 3.

Figure 5—Section of normal cat skin (H. & E. × 110).

Figure 6—Skin section 12 days post-inoculation; no treatment. Infection established intrafollicularly (P.A.S. × 110).



Figures 7-12—Skin sections. fig. 7) 19 days post-inoculation; no treatment. Skin thickening evident (P.A.S. × 110). fig. 8) 19 days post-inoculation; 7 days oral treatment. Skin thickening has decreased (H. & E. × 110). fig. 9) 30 days post-inoculation; 18 oral doses. Regression of dermatitis (H. & E. × 110). fig. 10) 49 days post-inoculation; 37 oral doses. Skin has returned to normal (P.A.S. × 110). fig. 11) 52 days post-inoculation; no treatment. Epidermal and dermal thickening evident (P.A.S. × 110). fig. 12) 65 days post-inoculation; 5 oral and 12 topical treatments. Regression of dermatitis (P.A.S. × 110).

*To face page* 105)

treatment but the bluish colour noted in the untreated animals was not at any time seen. However, from the 14th day of treatment, the affected hairs fluoresced only at their tips, giving a flecked appearance to the affected area under Wood's light.

Positive cultures of *M. canis* were recovered from all cats throughout the course of the experiment.

*Treatment experiment II*

The fluorescence, present along the entire length of the hairs at the beginning of this experiment, had gone from the base of the hairs in cats Nos. 1, 2 and 3 by the 5th day of treatment and on the 12th day there were only a few isolated fluorescing hairs to be seen. The animals were Wood's light negative in from 19-29 days (table 2).

| *CAT* | *Duration in days of;—* | | | |
|---|---|---|---|---|
| | *Clinical Signs* | *Positive Fluorescence* | *Positive Culture* | |
| 1 | 11 | 25 | 57 | Treated orally and topically and clipped on 9th and 16th day. |
| 2 | 11 | 19 | 43 | |
| 3 | 12 | 29 | 64 | |
| 4 | 79 | 127 | 127 | Control : never treated |
| 5 | 13 | 78 | 106 | Continuing observations on cats previously treated only orally. |
| 6 | 15 | 113 | 127 | |
| 7 | 13 | 78 | 120 | |

TABLE 2.—COMPARISON OF TREATMENTS WITH ORAL GRISEOFULVIN AND ORAL PLUS TOPICAL THERAPY AND HAIR CLIPPING.

The cats were clinically normal by the 12th day of treatment (fig. 3) but all 3 remained culturally positive for an average of 54 days after the first treatment (range 43-64 days). Cat No. 4, the control, still showed skin thickening and scaliness 25 days after the start of this experiment (fig. 4), *i.e.*, 79 days from the date of inoculation. There were also 2 strips, 2 cm. in length, of fluorescent hairs along the back.

Meanwhile, in cats Nos. 5, 6 and 7 which had been treated only by mouth, fluorescence had persisted for a mean duration of 90 days (range 78-113) and they gave positive cultures for a mean duration of 118 days (range 106-127).

## HISTOPATHOLOGY

The epidermis in the normal cat is very thin and it is difficult to distinguish strata. In the dermis the hairs are in groups, all projecting through a common follicular opening. The tissue surrounding the hairs is dense collagen and in this the majority of cells are fibroblasts. Small groups of smooth muscle fibres are located a short distance below the epidermis. These are the *arrector pili* muscles which are well developed in the cat (fig. 5). In the untreated animal 12 days after inoculation there were no clinical signs present but infection had been established (fig. 6) and fluorescence was evident. By the 19th day after inoculation the untreated cat showed hyperplasia of the epidermis including the follicle component. Vacuolation had occurred in the *stratum granulosum* and there was oedema in the dermis (fig. 7). The dermal reaction was mainly an increase in lymphocytes and histiocytes with some polymorphonuclear leucocytes including eosinophils. At this time, the treated animal which had received griseofulvin orally for 7 days with no other treatment showed that the established skin thickening had decreased (fig. 8). After 18 oral doses there was marked regression of the dermatitis (fig. 9) and after 37 oral doses, regression was complete (fig. 10). Meanwhile 52 days after inoculation the control cat showed heavy fungal infection of the follicles. Epidermal thickening was evident as was the dermal oedema (fig. 11). The dermal reaction showed an obvious increase in mast cells in addition to the usual inflammatory cells. This was the appearance at the end of the first experiment.

In the second experiment a cat which had received 5 oral doses and 12 topical treatments showed signs of regression of the dermatitis (fig. 12). At this time it was not possible to detect skin thickening clinically.

## DISCUSSION

Microsporosis in the cat is a mildly chronic inflammatory process with a protracted course. Although griseofulvin clears the infection in the hair follicle, it is some considerable time before the infected hair is shed. Thus the mean duration of clinical signs in 6 treated cats was 12·5 days as against 79 days in a control (table 3). However, the mean duration of fluorescence was 24 days, from commencement of treatment, in animals which received oral and topical griseofulvin and in which the infected hair was clipped, and 90 days in those receiving the drug only by mouth. Positive cultures were recovered for 55 days in those getting oral and topical treatment and having their hair clipped, and for 118 days in those receiving only oral griseofulvin. The control cat showed positive fluorescence and was culture positive for the duration of the experiment, a period of 127 days. Thus an important factor in the treatment of microsporosis in cats is the length of time that infected hairs remain on the animal prior to their being shed. Obviously these hairs constitute a public health hazard.

| | *Mean duration in days of:—* | | |
|---|---|---|---|
| | *Clinical signs* | *Positive Fluorescence* | *Positive Culture* |
| Oral and Topical Treatment+Clipping | 11 | 24 | 55 |
| Oral Treatment alone | 14 | 90 | 118 |
| Control | 79 | 127 | 127 |

TABLE 3.—SUMMARY OF RESULTS OF TREATMENTS OF *M. canis* INFECTION IN CATS.

These cats were kept in individual cages in a small room but in only one cat did a lesion (2 × 1 cm.) appear away from the inoculation site. In cat No. 7 the back cleared completely after 13 days treatment but 7 days later there appeared a small scab on a biopsy site over the sacrum. It was present for a month, showed positively fluorescing hairs, was positive culturally and healed spontaneously. The biopsy scar probably formed a focus for reinfection in this case. The protracted duration of positive fluorescence in cat No. 6 (113 days) was attributable to the presence of hairs embedded on the periphery of a biopsy scar.

It is clear that oral griseofulvin alone can affect a clinical cure but for a mycological cure the medication has to be assisted by the removal of infected distal parts of the hairs and by the use of an antifungal dressing.

## Résumé

La griséofulvine administrée par voie orale provoque la disparition de *M. canis* des folicules pileux de chats expérimentalement infectés.

Les manifestations cliniques disparaissent également bien que la fluorescence des poils persiste.

En coupant la partie distale des poils infectés et en appliquant une préparation antifongique on réduit la période de temps pendant laquelle s'observe la fluorescence et s'obtiennent les cultures du parasite.

## Acknowledgements

I am grateful to Glaxo Laboratories for supplies of griseofulvin and Messrs. A. Finnie and D. Boxall for photography.

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# DIRECT STAINING ON VINYL PLASTIC TAPE, DEMONSTRATION OF THE CUTANEOUS FLORA OF THE EPIDERMIS BY THE STRIP METHOD

Frances Keddie, Antoinette Orr and Dorothy Liebes

*Division of Dermatology, Department of Medicine, University of California Medical Center, Los Angeles 24, California, and the Palo Alto Medical Research Foundation, Palo Alto, California.**

The bacteria and fungi of the horny layer of the skin can be stained directly on Scotch brand tapes (#681 and #473) by ordinary histologic techniques. Methods are given for PAS, Gram, and Giemsa staining for light microscopy and for an acridine orange stain for fluorescent microscopy. Many other staining methods may be used. Additional uses are described, such as the examination of infected hairs, making of cultures from material collected on the tape, and a rapid staining method for immediate diagnosis.

The cheap and easily available transparent vinyl plastic tapes have proved to be chemically resistant to most routine histologic staining procedures, and, in consequence, strippings of the horny epidermis can be easily stained and permanently mounted through the use of these tapes. The surface epithelial cells, bacteria, and fungi adhere in a layer which is almost continuous and usually one cell thick. On successive strips from a single area, the horny layer and its contents can be entirely removed in serial layers. Although many staining methods can be used with the tapes, this paper reports only those especially useful for the study of bacteria and fungi.

## Materials and Methods

Ordinary †Scotch brand tape (#600) generally used to make strippings has a cellophane backing which is not resistant to water or chemicals. Not only does the cellophane film itself take up dyes, but the entire strip of tape curls up on contact with the water and other solvents used in histologic procedures. Newer Scotch brand tapes (#681 and #473) are made of vinyl plastic films which are more durable and considerably more resistant to moisture, chemicals and solvents. The adhesive of tape #681 tolerates dehydrating, clearing, and mounting in various resins; that of #473 is miscible only with glycerine-jelly.

Because of the improved ageing life of the vinyl plastic tapes, samples may be kept for several months at room temperature without deterioration of the tape or the specimen. Strippings are made in the usual fashion and are gently pressed to the hard smooth surface of plastic or glass.

Selected areas are cut apart with a knife and gently pulled away from the holder. The piece of tape can be held at the edge with forceps or can be transferred through the solutions in a ceramic (Gooch) crucible. Finally the tape is mounted with the sample, that is, the adhesive side of the tape toward the cover slip.

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* Supported by Grant #E-3928, National Institutes of Health.

† Samples of Scotch brand tape were provided by Minnesota Mining and Manufacturing Co., 6023 Garfield Avenue, Los Angeles, California.

### *Staining Procedures*

I. *Periodic acid Schiff (modified)*

1. Oxidize in 5% periodic acid 1-5 min.
2. Rinse in distilled water.
3. Immerse in Schiff's reagent 10-15 min. (Gomori, 1952).
4. Wash in running tap water 10 min.

Mount tape #473 in glycerine-jelly. For tape #681 complete the following:

5. Dehydrate in 3 changes of absolute alcohol.
6. Clear in 3 changes of terpineol.
7. Mount in balsam or HSR*.

*Results*: Fungi and bacteria stain deep red. Epithelial cells stain pale pink.

II. *Gram stain (Hucker modification) (Conn, Darrow & Emmel, 1960).*

1. Stain in ammonium oxalate crystal violet 1 min.
2. Wash in tap water.
3. Immerse in Gram's iodine solution 1 min.
4. Decolorize, with gentle agitation, in 95% alcohol 30 sec.
5. Rinse in tap water.
6. Counterstain with safranin 0, 0·25% solution 10 sec.
7. Wash in tap water.
8. Dehydrate, clear and mount as in I.

*Results* : Gram positive organisms stain deep violet, Gram negative organisms stain orange; epithelial cells stain pale brown and orange.

III. *Giemsa Stain.*

Staining solution : Giemsa† solution diluted 1:9 parts of water.

1. Stain in Giemsa solution 3 min.
2. Wash in distilled water.
3. Dehydrate and clear as in I.

*Results* : Metachromatic staining of fungi, bacteria and epithelial cells stain pink and blue.

IV. *Acridine Orange Stain‡ (Bertalanffy, 1960).*

Staining solution : Aqueous solution of acridine orange 0·1% is diluted to 0·01% with phosphate buffer pH 6·0.

1. Fix in absolute alcohol 1 min., dip in 1% acetic acid solution, and then in distilled water.
2. Stain in acridine orange solution 3 min.
3. Remove excess stain in phosphate buffer 1-2 min.
4. Differentiate in 1/10 molar calcium chloride 1-2 min.
5. Dehydrate, clear and mount in HRS or other non-fluorescent synthetic mounting medium.

*Results* : By fluorescent microscopy the fungus elements and epithelial cells stain bright green and orange.

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* Harleco synthetic resin ; Hartman-Leddon Co., Philadelphia, Pa.

† "Giemsa's Stain Solution," National Aniline Division, Allied Chemical Corp., New York 6, N.Y.

‡ "Acridine Orange C.I. 46005," National Aniline Division, Allied Chemical Corp., New York 6, N.Y.

### *Quick Staining Methods for Immediate Diagnosis*

Stain a piece of transparent Scotch brand tape stripping (#473, #600, #681 or #800) by placing the tape, adhesive side up on a slide and dropping on the stain. Stain 1 min. with Hucker crystal violet, Leoffler methylene blue or Giemsa solution. Rinse quickly with alcohol, blot dry and mount in balsam, a synthetic mounting fluid or glycerine-jelly. All sections are usable for several hours and some will last indefinitely.

*Note* ; *n*-butyl alcohol is better than ethyl or isopropyl alcohol for dehydration, because with it, the plastic film remains quite clear.

Terpineol softens the adhesive less than does xylene, though both are acceptable. Permount may, in time, cause a faint haziness of the plastic film; preparations appear to stay clear in HSR and in balsam. In table I are listed the various kinds of tape and the agents which they will tolerate during dehydrating, clearing and mounting. For fluorescent-antibody staining it was found desirable to separate the cells from the tape. This was done by the following steps :

| *Scotch-brand Tape #* | *Dehydration* | *Clearing* | *Mounting* |
|---|---|---|---|
| 681 | *n*-butyl alcohol | terpineol | Harleco synthetic resin balsam |
| 473 | none | none | glycerine-jelly |
| 800 | isopropyl, ethyl, or *n*-butyl alcohol | xylene<br>terpineol | Balsam |
| 600 | ethyl alcohol | blot dry | Permount |

TABLE 1.—AGENTS PREFERRED FOR THE MAKING OF PERMANENT PREPARATIONS.

1. Gelatine slides by dipping in 1% calf-skin gelatin and allow to dry. Fix the gelatin in 1% formol solution and allow to dry.
2. Apply the sample to the surface of the gelatinized slide with firm pressure. Place in carbon tetrachloride for 24 hours. This will soften the adhesive, thus allowing the plastic backing to peel off and leave the sample attached to the slide. Staining procedures can then be carried out in the usual way.

## DISCUSSION

Jan Wolf (1939) stained some of the horny cells after removing them from the adhesive of the tape ; however, he was primarily interested in the surface-relief of the cells which were adherent to the tape. Pinkus (1951) demonstrated that the whole of the horny layer could be removed in 20 to 30 layers, each one cell

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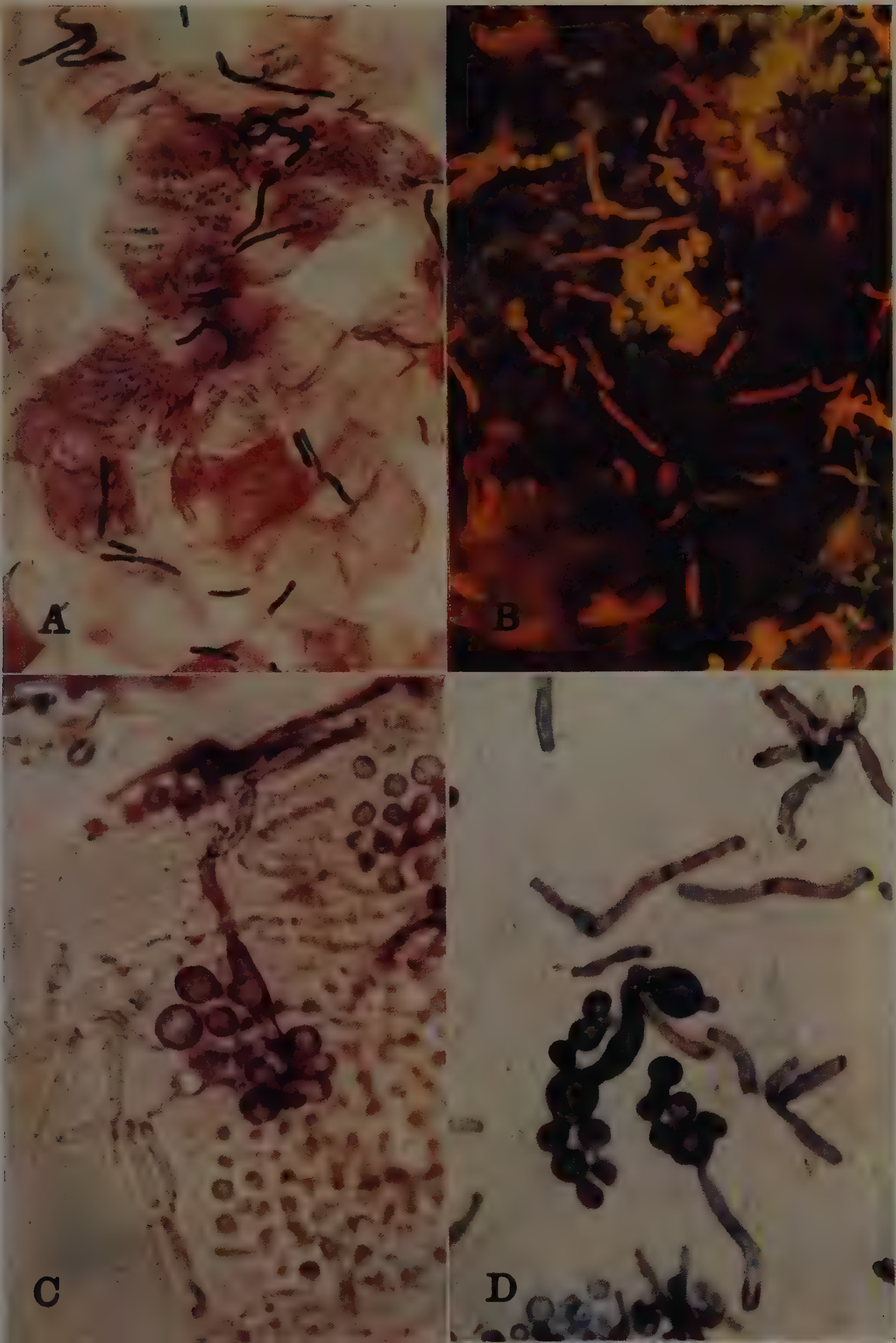
## LEGEND

Plate 1.—Scotch brand (#681) tape strips from tinea versicolor. Note that the elements of *Malassezia furfur* are maintained in their normal relations by the tape. (A) Gram stain ×580. (B) Acridine Orange stain ×650. (C) PAS stain ×1360. (D) Giemsa stain ×1360.


A
B
C
D

thick, by successive strippings on Scotch brand tape #600. Porto (1953) as well as Vanbreuseghem (1954) applied Wolf's method to find and study *Malassezia furfur* in tinea versicolor scales. Porto preferred unstained material. No clearing or mounting agents were used; the tape was applied directly with the adhesive against a glass slide. Vanbreuseghem, on the other hand, stained the skin of the patient with tincture of iodine or eosin before stripping off the scales and thus improved the contrast in his preparations.

Attempts made in our laboratory to stain specimens on ordinary Scotch brand cellophane-backed tapes failed. With the acetate film tapes (Scotch brand tape #800 in particular) it was possible to stain the strippings in aqueous solutions and mount them in balsam after a quick passage through alcohol and xylene. Such sections have remained well stained and clear after 2 years. The films of the newer Scotch brand tapes are made of vinyl plastic which is unaffected by most histologic procedures, and of these #681 has been the most satisfactory. The adhesive of tape #681 is as aggressive as that of the ordinary tape #600, but neither is sticky enough to strip the scales from greasy, moist skin, nor from the palms or soles. It will, however, remove resting vellus hairs, and will hold loose animal and human hairs for staining. Cultures of bacteria and fungi of the horny layers can be made by applying the tape strippings to the surface of appropriate culture media. It is possible to stain the tape after incubation on the culture media. For fluorescent microscopy tape #681 strippings are stained and mounted in non-fluorescent synthetic mounting fluid or in glycerine.

## Résumé

Les bactéries et les champignons de la couche cornée peuvent être facilement colorés dans les prélèvements effectués par la technique du "scotch-tape" (rubans n° 681 et 473). Parmi les diverses méthodes de coloration qu'il est possible d'utiliser, on emploie les techniques de Hotchkiss-Mac Manus, de Gram et de Giemsa, en lumière normale et, en lumière ultra-violette, l'orangé d'acridine.

D'autres applications de cette méthode, comme par exemple l'examen direct des cheveux parasités, les cultures à partir d'échantillons prélevés de la couche cornée, ainsi qu'une méthode rapide de coloration pour le diagnostic sont décrites.

## Acknowledgement

We wish to thank Mr. G. F. Orr for contributing mycological material for this project.

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# PURIFICATION OF THE SPHERULE-ENDOSPORE PHASE OF *COCCIDIOIDES IMMITIS*[*]

H. B. Levine

*Naval Biological Laboratory, School of Public Health,*
*University of California, Berkeley, California.*

Upon shaking an aqueous suspension of spherules, endospores and mycelium of *Coccidioides immitis* with chloroform, selective agglomeration of the mycelium around the emulsified chloroform droplets occurred. The mycelial strands became aligned on the surface of the droplets in parallel configurations, as an apparent consequence of surface forces, and the droplets accumulated at the chloroform-water interface. Spherules and endospores were not so-affected and could be resuspended readily in the aqueous phase of the system. This observation formed the basis of a procedure that was employed to obtain the spherule-endospore phase from cultures of *C. immitis* in highly purified form.

In studies on the comparative immunogenicity of the morphologic phases of *Coccidioides immitis*, Levine, Cobb & Smith (1960, 1961) demonstrated that superior protection was afforded to mice by vaccination with the spherule-endospore phase of growth than that afforded by vaccination with equal doses of either the arthrospore or mycelial phases. The design of the experiments required that each of the above-mentioned morphologic phases be procured in a state of high purity since antigenic cross-reactivity could be presumed to exist. It was noted during these studies that whereas the preparation of relatively pure suspensions of arthrospores or mycelium was accomplished readily, difficulties were encountered in the purification of the spherule-endospore vaccine.

Cultures containing spherules and endospores invariably contained appreciable numbers of mycelial elements. The latter could be removed by filtration, but, in so-doing, more than 90% of the spherules were lost by entrapment in the entangled mycelium. Losses were also high in spherule preparations purified by sedimentation through dense sucrose solutions.

Recently I have found that very pure preparations of spherules and endospores could be obtained from suspensions contaminated grossly by mycelium by a simple procedure utilizing chloroform emulsions. Washed organisms, suspended in water, were shaken with chloroform and, after a period of settling, the fungal material accumulated at the chloroform-water interface. The spherules were released into the aqueous phase by gentle rotary agitation and this phase was then removed by pipette. As will be described, the mycelium was held rather tenaciously at the interface, apparently by surface forces, and, after several extractions of the interface, relatively few spherules but most of the mycelial structures were left at the interfacial site.

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[*] This work was sponsored by the Office of Naval Research and the Bureau of Medicine and Surgery, United States Navy, under a contract between the Office of Naval Research and the Regents of the University of California. Reproduction in whole or in part is permitted for any purpose of the United States Government.

## Methods and Results

Spherule-endospore structures from *C. immitis* strain Silveira were grown in the defined medium of Converse (1957) or in a medium containing 0·4% Casamino acids (Difco, Detroit) and 0·4% glucose. The conditions of inoculation, aeration and incubation have been described elsewhere (Levine, Cobb, & Smith, 1960).

At the end of the incubation period, 0·5% formalin (final concentration, vol./vol.) was added to the cultures and allowed to remain in contact with the organisms for a minimum of 8 days. During this time the contents of the culture vessels were twice transferred to other vessels to assure that no organisms on the upper walls of the vessels escaped sterilization. The sterility of the suspensions was ascertained by cultural procedures.

In a typical experiment, 1 l. of the fungal suspension was filtered through 8 layers of surgical gauze to remove the large clumps of mycelium. The organisms were then harvested by centrifugation (1000 × G, 20 min.), thrice washed in sterile distilled water by this procedure, and finally resuspended uniformly in 250 ml. water. It was determined that the washing procedure was essential for the subsequent separation of the morphologic phases.

The preparation, which was comprised of spherules, free endospores and mycelium (fig. 1A), was transferred to a glass-stoppered Erlenmeyer flask and 500 ml. of chloroform were added. The flask was then shaken vigorously for 10 minutes and was allowed to stand with intermittent swirling for an additional 20 minutes until the mixture was clearly partitioned into a lower chloroform layer, a clumped gelatinous-appearing interface and a uniformly turbid upper layer. After further gentle rotary agitation, the upper layer was removed by pipette and examined microscopically.

When cell harvests that had been grown in Converse's medium were used, the upper layer contained a ratio of approximately 1 short hyphal strand to $10^3$ spherule and endospore particles (fig. 1B), however, the relative concentration of mycelial elements was approximately 10-fold higher when the procedure was used with cells harvested from the Casamino acid medium. The interface, containing organisms from either medium, was then extracted 3 times by replacing the removed aqueous phase with an equal volume of sterile distilled water into which spherules and endospores were released by rotary agitation as described above.

No further treatment was required for the partition of organisms grown in Converse's medium; the residue remaining at the interface consisted primarily of mycelium (fig. 1C) indicating that efficient separation was accomplished. However, additional steps were necessary to achieve a comparable state of purity in the spherule-endospore suspensions obtained from the Casamino acid medium. The cell content of the pooled upper layers was harvested by centrifugation (1000 × G, 20 min.), resuspended in 20 ml. water and transferred to a 30 × 200 mm. test tube. After 20 ml. of chloroform were added, 5 ml. of 1% (vol./vol.) aqueous acetic acid were added and the mixture was shaken and partitioned as above, followed by 3 extractions of the interface with acidified water. The purity of the spherule-endospore suspension was then comparable to that obtained with organisms grown in Converse's medium and partitioned as described. Acidification was shown to favour the separation of Casamino acid-grown organisms, however it is possible that this step could be omitted if repeated partitions were made in the absence of added acid.

Minor skills, readily achieved with experience, were required for the success of the procedure; after separation of the chloroform-water mixture, excessive agitation released some mycelium as well as spherules and endospores into the upper aqueous phase. Also, mycelial structures were found during removal of the aqueous phase as the pipette approached close to the interface. In my early experience it was desirable to examine microscopically each aliquot removed from the aqueous phase before pooling it with previously-removed aliquots.

Microscopic inspection of the interfacial emulsion suggested that the mycelium was retained at this site by surface forces. There were numerous suspended droplets of chloroform in water at the interface and around each of these droplets mycelial strands were aligned in essentially parallel serpentine configurations (fig. 1D). As the microscope was focused into different planes, it appeared that the mycelium resided on the surface of the droplets. Seldom were spherules or endospores seen adhering to the emulsified droplets suggesting that these latter structures differed markedly in surface properties from those of the mycelium. After rupture of the droplets on the microscope slide, the mycelium retained its characteristic alignment (fig. 1C) which was in marked contrast to the random entangled orientation of mycelium (fig. 1A) in untreated suspensions.

## Discussion

Although results on the immunogenicity of spherules purified by the described chloroform partition method will not be available for some time, I consider the methodology worthy of report because of its possible application in other mycologic problems. Further, the results indicate that there are physical and probably chemical differences between the cell wall of the mycelium and that of the spherules and endospores; this aspect may be of particular relevance to vaccine studies since the immunogenicity of the spherule-endospore structures appears to reside primarily in the cell wall fraction (Levine, Cobb & Smith, 1961).

The attraction of micro-organisms to the interface of immiscible fluids has been well-described by Mudd & Mudd (1924a, b) and by Reed & Rice (1931). The observation was then made that acid-fast bacteria entered the oil phase of a water-oil emulsion but that other bacteria remained either at the interface or in the aqueous phase. It was postulated that the non-polar fatty content of the acid-fast bacteria prevented their being wetted by the water but allowed them to be wetted by the non-polar oil. Reed & Rice (1931) demonstrated with a number of acid-fast and non-acid-fast bacteria that essentially similar separations occurred in biphasic systems consisting of water and either carbon tetrachloride, ether, xylol, kerosene, benzene, petroleum, chloroform, carbon bisulphide and butyl, amyl or iso-amyl alcohols. In the coccidioidal model, however, separation of the mycelium did not occur in amyl or butyl alcohol-water emulsions. The addition of methanol, ethanol or propanol to the chloroform-water system interfered with the rather selective retention of mycelium at the interface.

The emulsification of chloroform in an aqueous suspension of spherules, endospores and mycelium on a microscope slide caused an initial clumping of the mycelium, presumably around micro-droplets of chloroform. Apparently such clumps, in the system used for separating the mycelium, had increased density because of their chloroform content and therefore settled to the interface. The clumps were not dislodged by the small amount of energy required to

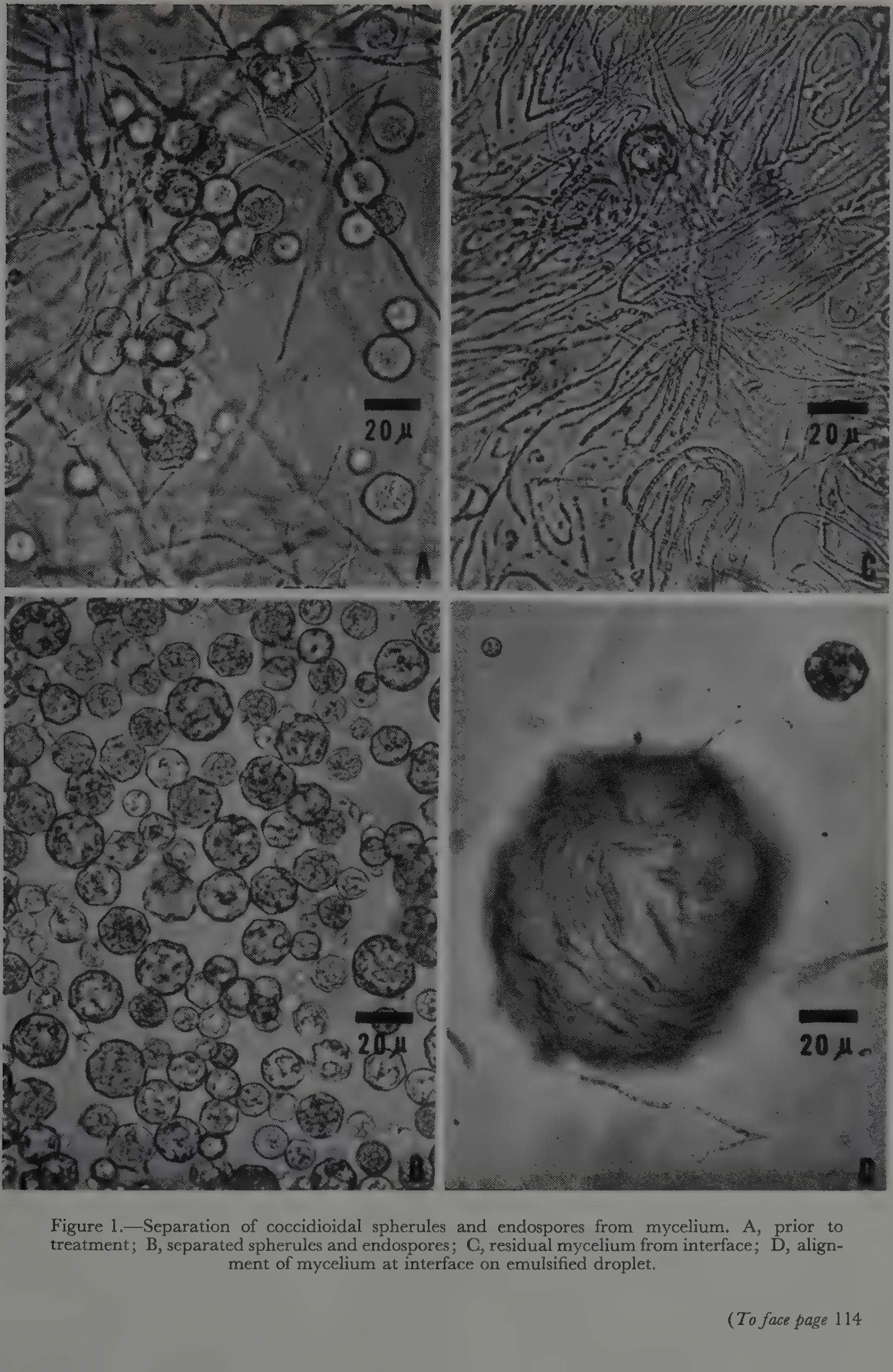

Figure 1.—Separation of coccidioidal spherules and endospores from mycelium. A, prior to treatment; B, separated spherules and endospores; C, residual mycelium from interface; D, alignment of mycelium at interface on emulsified droplet.

suspend the unclumped spherules and endospores in the aqueous phase. In this regard, the mycelium may have been flocculated in a manner analagous to the flocculation of bacterial cells by hydrophilic colloids (Hodge & Metcalfe, 1957; Klein, Metcalfe & Lincoln, 1958). However, the nature of the forces governing the affinity of the mycelium for the surface of chloroform droplets has not been elucidated by this study.

## RÉSUMÉ

L'addition de chloroforme à une suspension de sphérules, d'endospores et de mycélium de *Coccidioides immitis,* provoque, après agitation, une agglomération du mycélium autour des gouttelettes de chloroforme. Les filaments mycéliens s'alignent parallèlement à la surface des gouttelettes, probablement du fait des forces de tension superficielle, et les gouttelettes s'accumulent à l'interface du chloroforme et de l'eau. Les sphérules et les endospores peuvent facilement être remises en suspension dans la phase aqueuse du système.

Cette observation a été à l'origine d'une méthode employée pour séparer la phase sphérules-endospores des cultures de *C. immitis* sous une forme très purifiée.

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# THE PRODUCTION OF TRICHOPHYTIN ANTIGEN IN SUBMERGED CULTURE OF *TRICHOPHYTON MENTAGROPHYTES*

R. C. Codner, C. N. D. Cruickshank, M. D. Trotter & S. R. Wood

*Medical Research Council Unit for Research on the Experimental Pathology of the Skin,*
*The Medical School, University of Birmingham, Birmingham, 15*
*and*
*Medical Research Council Antibiotics Research Station,*
*4 Elton Road, Clevedon, Somerset*

A method is described for the production in submerged culture of large amounts of crude antigenically active material, trichophytin, from *Trichophyton mentagrophytes.* The crude material is sufficiently free from irritant properties to be used clinically and may be further purified for investigation of the relationship between the chemical structure and biological activity of trichophytin.

Extracts of the mycelium of fungi of the *Trichophyton* group—"trichophytins"—may be used by intradermal injection to test for skin hypersensitivity of patients infected with these organisms. Although varying in detail, the current methods of producing trichophytin follow that most recently described by Sulzberger (1940). The fungus is grown for 6-8 weeks on the surface of a shallow layer of fluid medium, the mycelium is removed, ground up in an aliquot of the medium and passed through Seitz or Berkefeld filters. The filtrate contains the active trichophytin. Phenol (0·5%) is added as preservative.

Previous studies (Cruickshank, Trotter & Wood 1960; Barker & Trotter 1960) had shown that a purified trichophytin could be obtained by extracting the mycelium from surface cultures of *Trichophyton mentagrophytes* with ethylene glycol and that the use of this material, instead of a crude extract, offered several advantages. Surface culture, however, is extravagant of time and space and so experiments were made to find out whether *T. mentagrophytes* could be grown successfully in deep culture and whether a trichophytin suitable for testing was produced under these circumstances.

## Methods

### *Shake flask cultures*

Shake flask cultures were made in 20 oz. medical flat bottles each containing 75 ml. of medium and an inoculum of 2 ml. of mycelium and medium obtained by shaking up, by hand, a 14 day old surface culture on liquid medium. In order to obtain maximum aeration using a reciprocating shaker, the bottles were shaken on edge at an angle of 25° to the tray of the shaker and in line with the throw. The shaker made 100 excursions/min. in each direction and the throw (about $1\frac{1}{2}$ in.) was adjusted so that the medium just failed to enter the neck of the bottle.

*Laboratory fermenter*

A laboratory scale fermenter (16 l. working volume) based on a 20 l. Pyrex aspirator bottle, was used (fig. 1). The aspirator (a) was equipped with a stainless steel paddle stirrer (s) 2½ in. diam. with 2 flat blades, set at 45° to the shaft line, to each of which were attached three 1 in. lengths of stainless steel chain. The shaft of the stirrer passes through a brass bushed bearing and gland (g) held in the neck of the aspirator by a rubber bung (b). On the outside of the gland unit, detailed construction of which is shown in inset Y, was a tubular extension (e) to which a vacuum line (v) was attached via a wash bottle train (t) containing disinfectant. This served to trap any organisms which might have escaped through the gland.

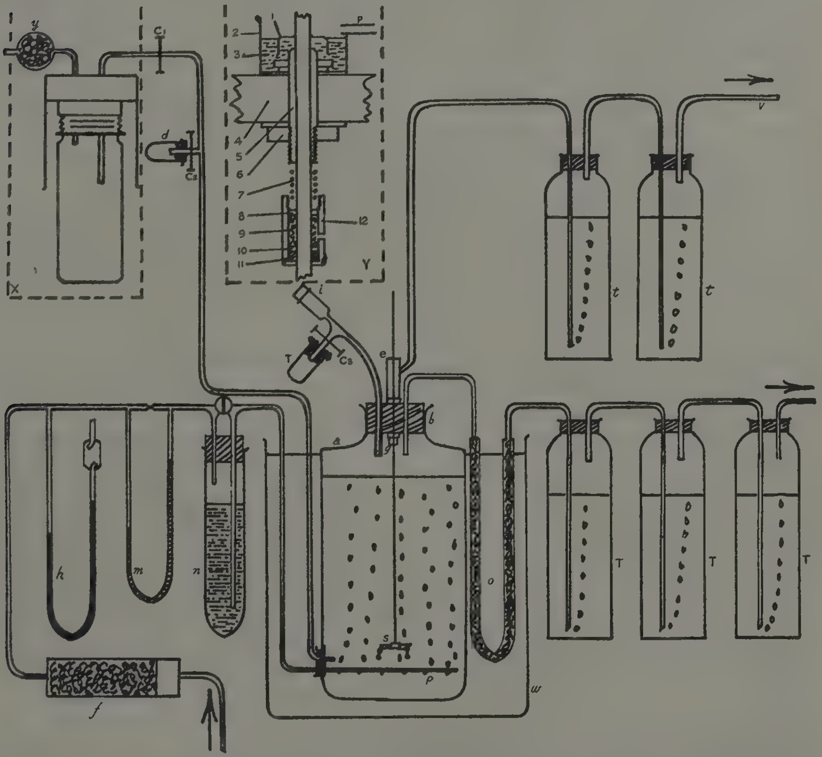

Figure 1.—Diagram of apparatus used for trichophytin production in submerged culture:—a—20 l. Pyrex aspirator; b—bung; $c_1$, $c_2$, $c_3$—screw clips; d—connexion for harvesting filter; e—tubular extension from stirrer bearing; f—inlet air filter; g—gland of which construction details are shown in inset Y; h—mercury manometer; i—inoculation point; m—capillary-manometer flowmeter; n—antifoam reservoir; o—outlet airfilter; p—sparge pipe; r—connexion for recycling from harvesting filter; s—stirrer paddle; T and t—wash bottle trains containing disinfectant; v—vacuum line; w—water bath. Inset X shows details of sampling device including McCartney bottle and air filter y. Inset Y shows construction of stirrer bearing and gland. 1—shaft cup; 2—tubular extension e; 3—oil; 4—bung b; 5—brass bushed stirrer shaft bearing; 6—bung compression collar; 7 compression spring; 8 and 10—washers; 9—greased asbestos packing; 11—felt washer; 12—gland sleeve.

Through the lower tubulature of the aspirator was led a perforated sparge pipe (p) for introduction of air and a sampling tube connected by rubber tubing with a screw clip ($c_1$) to a McCartney bottle equipped with a second tube carrying a small air filter (y). The ingoing air was sterilized by passage through a cotton wool packed air filter (f) 3 in. diam. by 14 in. long and a capillary manometer type flowmeter (m) was connected in the ingoing air line as was a device for addition of antifoam (n) during the fermentation run. The ingoing air pressure was maintained on the mercury manometer (h) at the value for which the flowmeter (m) was calibrated.

An inoculator port (i) with side connexion (r) was fitted through the bung in the neck and this bung also carried the air outlet tube which leads to a second smaller cotton wool filter (o) to remove entrained droplets and fungus particles. As a further precaution the exhaust air was passed through a second series of wash bottles (T) containing disinfectant solution.

The whole aspirator was, during the fermentation, immersed up to the shoulder in a waterbath (w) held at 28°.

The aspirator with the above attachments was sterilized by autoclaving for 15 minutes at 15 pounds/sq. in. steam pressure. In order to sterilize 16 l. of medium in bulk by autoclaving without stirring, prolonged heating would be needed to ensure heat penetration. This was avoided by sub-dividing the medium into 16 × 1 l. quantities in 2 l. flasks each with a bung, air filter and transfer tube dipping to the bottom of the flask. The transfer tubes from the blocks of eight flasks were connected up to one outlet point by rubber tubing with screw clips and appropriate glass T-pieces. These flasks were sterilized by autoclaving for 10 minutes at 10 pounds/sq. in. and their contents transferred to the aspirator by making an aseptic connexion from each block of 8 flasks in turn to the inoculator port and blowing over the contents of each flask by connecting an air line to the filter of each flask in turn with the corresponding screw clip open. After the medium had been transferred to the aspirator, the aspirator was placed in the water bath and the air lines were connected.

The aspirator was inoculated with 2 surface cultures of *T. mentagrophytes* (NTC D281) grown in 20 oz. medical flat bottles. These surface pellicles were broken up by shaking and transferred aseptically to a transfer bottle with hooded pipette. The aspirator was inoculated from the transfer bottle by connecting a hooded pipette to the inoculator port (i).

The aspirator was incubated at 28° and stirred at 1000 r.p.m. A continuous airflow of 15 l./min. was maintained. The usual fermentation time was 7 days. Before autoclaving 50 ml. of a 1:5 solution of Silicone Antifoam A (Hopkin & Williams Limited, Freshwater Road, Chadwell Heath, Essex) in chloroform was added to the empty aspirator. A further 3% solution of antifoam in white oil was added as required during the run.

### *Culture media*

Experiments were made with the following culture media:—(a) M.P; Malt extract (Munton & Fison Limited, Cedars Factory, Stowmarket, Suffolk) 4%; Panmede (Paines & Byrne Limited, Greenford, Middlesex) 1%; Distilled water to 100%. (b) G.P.C; Glucose 4%; Panmede 1%; Acid hydrolysed casein (Oxoid) 2%; Distilled water to 100%.

### *Harvesting*

Various harvesting methods were used. In the shake cultures this presented no problems and mycelium and culture fluid were separated by centrifugation. After drying in 3 changes of acetone and grinding in a mortar the mycelium was extracted with ethylene glycol and the debris removed by centrifugation and the supernatant dialysed, Seitz filtered and freeze-dried. The supernatant medium was Seitz filtered, dialysed and freeze-dried and weighed.

Harvesting from the fermenter was more complicated. In order to flocculate the mycelium the pH of the fermentation was adjusted by addition of hydrochloric acid, to pH 6 approximately. A sample of known volume was withdrawn and titrated to this pH and the calculated volume of acid was then added to the whole fermentation. A slurry of Kieselguhr (160 g.) in water was also added to the fermentation to facilitate filtration.

Filtration was carried out with a cloth bag filter supported in a wire mesh cage; the whole being enclosed in a length of 4 in. industrial glass pipe. The filter inlet was attached to connection (d) and provision was made to recycle the filtrate through a peristaltic pump back to the fermentation via connexion (r) with clips ($c_2$) and ($c_3$) open until the filtrate appeared bright. The bright filtrate was then collected in calibrated 20 l. aspirators fitted with stirrers and air filters, via a closed system of rubber tubing. The filtrate in the collecting aspirators was precipitated with 3 volumes of acetone, added with constant stirring.

The mycelium collected in the filter bag was dehydrated with acetone by filling up the length of industrial pipe with acetone and allowing the whole to stand overnight before dismantling. The mycelium was removed from the filter bag and resuspended in further acetone before collection on a Buchner filter on which as much acetone as possible was removed by suction.

The precipitate formed by acetone treatment of the broth filtrate was recovered in a small Sharples centrifuge or by filtration and finally washed with fresh acetone and sucked dry on a Buchner filter.

In some batches the acetone was added to both mycelium and culture fluid to render filtration easier. Final preparation of purified trichophytin was made by extracting either the acetone dried mycelium or the acetone precipitated material from the culture medium with ethylene glycol, dialysing and freeze-drying.

### *Biological testing*

Tests for the activity of the various samples were made on sensitised and normal guinea pigs as described by Cruickshank, Trotter & Wood (1960). All tests on guinea pigs were carried out at a concentration of 1 mg./ml. in normal physiological saline. Final tests of some of the materials were made on human volunteers at a concentration of 0·1 mg./ml. in normal physiological saline. Only the delayed (tuberculin-type) reactions were considered.

## RESULTS

### *The growth of Trichophyton mentagrophytes in the fermenter*

The first fermentation in the 16 l. laboratory fermenter was run for 11 days with M.P. medium, an aliquot having been extracted at 4 and 7 days. Typical macrospores and smaller bodies which could be interpreted either as oidia or microspores were present at 4 days and after. Figures 2 and 3 show the general appearance of growth and macrospores at 96 hours in this run. By 11 days considerably autolysis had set in. Subsequent runs were harvested at 7 days and although the growth of the organism showed the same general pattern the following characteristics varied from one fermentation to another: macrospores although always present varied in numbers, the degree of torulose swelling of the mycelium differed between one fermentation and another, and there was in some fermentations an initial lag phase of some 2 days when only small germlings could be found while in other fermentations this lag phase was absent. These variables did not appear to be correlated one with another. Figure 4 shows mycelium in G.P.C. medium at 42 hours before macrospore formation or torulose swelling is evident while fig. 5 shows marked torulose swelling present in another G.P.C. fermentation at 72 hours. The growth rate in G.P.C. medium tended to be faster than in the M.P. medium. In either medium the pH rose to between 7 and 8 in 7 days.

*Yield of active material*

The yields obtained in shake flask cultures (18-20 oz. flasks containing 75 ml. medium), using the M.P. medium and the G.P.C. medium and harvested after 7 days incubation, are shown in table 1. Medium M.P. which includes malt provided a slightly smaller weight of mycelium but about the same amount of glycol extract. The solid materials obtained by freeze-drying the dialysed supernatants were approximately the same weight. About 20 mg. active extract were obtained per 100 ml. of medium.

| *Medium* | *Culture volume (ml.)* | *Weight of mycelium (g.)* | *Weight of Supernatant dialysed and freeze dried (g.)* | *Weight of glycol extract of mycelium (g.)* |
|---|---|---|---|---|
| M.P. | 1250 | 7·4 | 2·8 | 0·22 |
| G.P.C. | 1250 | 12·7 | 2·4 | 0·17 |

TABLE 1.—YIELDS OF MYCELIUM AND EXTRACTS OF *Trichophyton mentagrophytes* WHEN GROWN IN SHAKE CULTURE USING M.P. AND G.P.C. MEDIA.

Biological tests (table 2) showed that the most active glycol extract came from the mycelium grown in the G.P.C. medium and least from the supernatant of this medium. It appeared as if in the G.P.C. medium less of the activity diffused into the medium than in the M.P. medium.

| *Guinea pig* | *Lesion diameter (m.m.)* | | | |
|---|---|---|---|---|
| | *M.P. glycol extract of mycelium* | *M.P. Supernatant* | *G.P.C. glyool extract of mycelium* | *G.P.C. supernatant* |
| Control | 4 | 5 | 4 | 0 |
| „ | 4 | 5 | 4 | 0 |
| Sensitized | 6 | 9 | 9 | 7 |
| „ | 20 | 16 | 26 | 12 |
| „ | 29 | 15 | 28 | 18 |
| „ | 13 | 13 | 15 | 5 |
| „ | 15 | 14 | 20 | 15 |
| Mean lesion diameter on sensitized animals | 16·6±3·8 | 13·4±1·2 | 19·8±3·5 | 11·4±2·8 |

TABLE 2.—BIOLOGICAL ACTIVITY OF EXTRACTS OF *Trichophyton mentagrophytes* WHEN GROWN IN SHAKE CULTURE USING M.P. AND G.P.C. MEDIA.

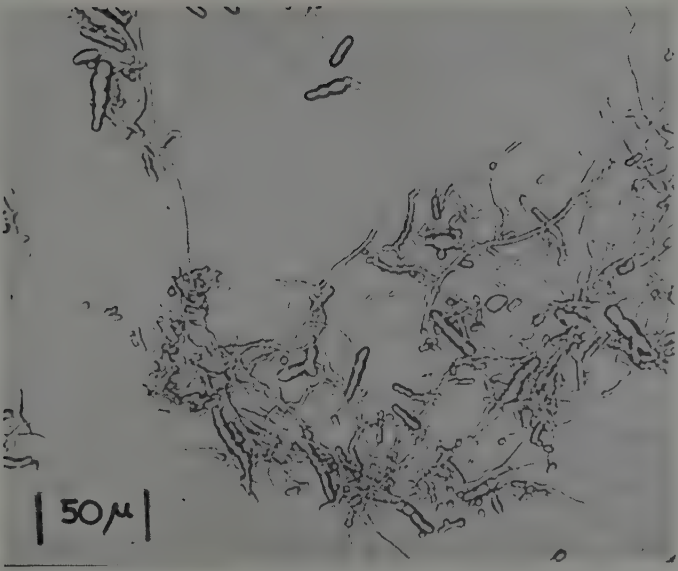

Figure 2.—Growth of *Trichophyton mentagrophytes* in submerged culture. M.P. medium 96 hours, unstained.

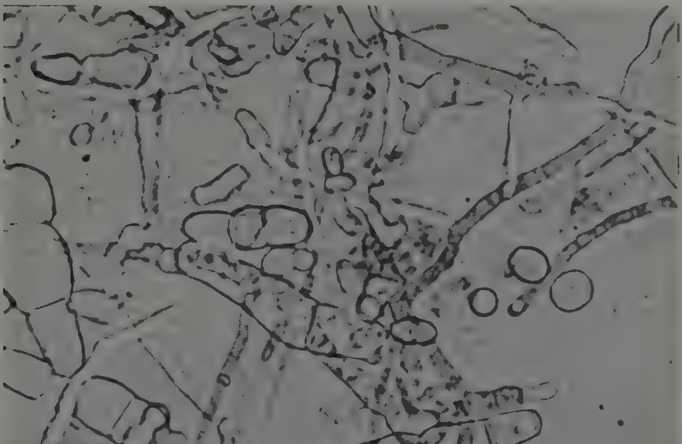

Figure 3.—*T. mentagrophytes* as fig. 2 showing macrospores and microspores.

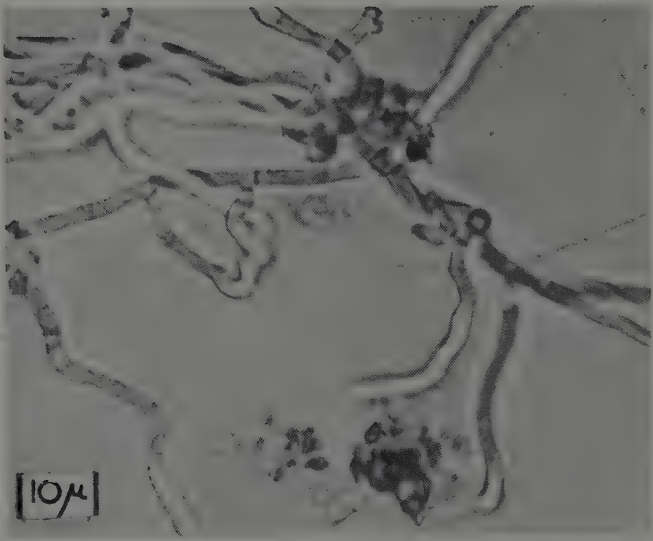

Figure 4.—*T. mentagrophytes* submerged culture in G.P.C. medium (Batch 3). Unstained, 42 hours, young undifferentiated hyphae.

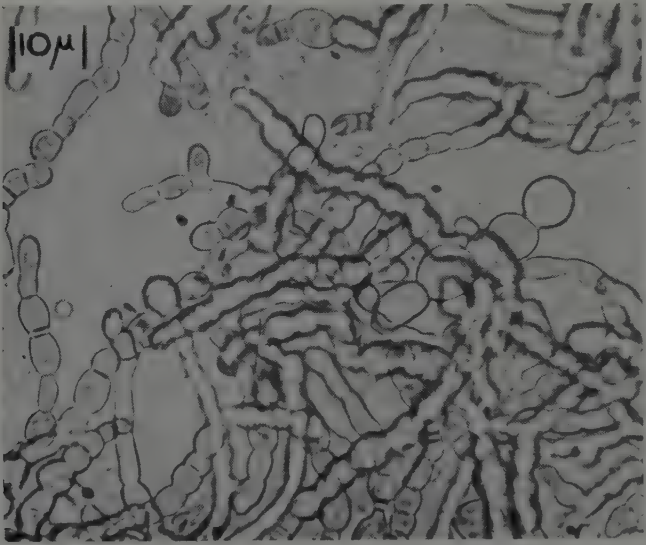

Figure 5. *T. mentagrophytes* submerged culture in G.P.C. medium (Batch 6). Unstained, 72 hours, macrospores and torulose hyphae.

The average yield of purified trichophytin from the laboratory fermenter was about 70 mg. per 100 ml. of medium—about 3 times that from the shake flask cultures. As shown in table 3 this produced slightly larger lesions on sensitised guinea pigs but no valid comparison can be drawn between mean delayed reactions on different batches of guinea pigs. No material from 7 day culture caused any non-specific reaction but samples from the 11 day run which had shown some autolysis gave rise to diffuse dusky irritant lesions on control animals.

| *Medium* | *Weight of mycelium* (*g.*) | *Weight of glycol extract* (*g.*) | *Mean lesion diameter* (*mm.*) ± *SE* |
|---|---|---|---|
| M.P. | 162 | 11·6 | 19 ± 0·7 |
| M.P. | 161 | 11·3 | 24 ± 1·4 |
| G.P.C. | 416 | 9·9 | 22 ± 2·1 |
| G.P.C. | 542 | 12·0 | 18 ± 2·6 |

TABLE 3.—THE YIELDS OF ACTIVE MATERIALS FROM THE LABORATORY FERMENTER.

Table 3 shows that in the laboratory fermenter a greater weight of mycelium was produced in the G.P.C. medium than in the M.P. medium but that the amount of active material was about the same.

As with the shake flask cultures active material could be precipitated from the culture fluid by adding acetone. For example, 150 ml. of the culture fluid yielded 93 mg. of precipitate on adding one volume of acetone, 160 mg. with the second volume and 33 mg. with the third. These fractions were each tested on only 2 guinea pigs and gave reactions of 20 and 13 mm., 23 and 18 mm. and 0 and 0 mm. respectively. Thus the bulk of the active material can be precipitated by adding two volumes of acetone.

## DISCUSSION

The main object of these experiments was to obtain large amounts of semi-purified trichophytin which could subsequently be fractionated to produce a chemically pure "trichophytin" the structure and activity of which could be further investigated.

It is likely, however, that submerged culture may prove to be an economic method of producing trichophytin for clinical purposes. For example, a 16 l. fermentation run for 1 week may produce 10 g. of partially purified material, sufficiently free of irritant properties for it to be used clinically. Since the appropriate concentration of this material for skin testing in humans is about 0·1 mg./ml., the volume of testing solution from 1 fermentation would be 100 l. or sufficient to test 2 million people using injections of 0·05 ml. The material may be kept indefinitely in the dry state thus ensuring reproducibility of results. The yields of acetone dried mycelium and of partially purified trichophytin per litre of medium from the laboratory fermenter are about the same as that obtained from surface cultures, but the saving in space and time afforded by the former method is a very great advantage.

The 2 culture media used gave closely similar results. Medium G.P.C., however, has the advantages of containing no polysaccharides and being dialysable. M.P. medium contains a proportion of polysaccharide derived from the malt which might conceivably give rise to false reactions in certain individuals and which make final purification more difficult.

It will be noted that in deep culture a considerable amount of active material can be obtained from the medium. This is not so with surface cultures. It is important, however, that the fermentation is stopped before autolysis occurs as under this circumstance toxic materials are liberated into the culture medium.

Although emphasis has been placed upon the production of a partially purified trichophytin the submerged culture method may also be used to produce the classical type of test material by grinding the mycelium in the medium. There is the risk, however, of producing too potent a trichophytin and so standardisation by the guinea pig test is desirable.

## Zusammenfassung

Beschreibung einer Methode für die Herstellung von antigenetisch aktivem Rohmaterial aus *Trichophyton mentagrophytes* (trichophytin) im Submersverfahren.

Das Rohmaterial ist hinreichend frei von irritierenden Eigenschaften, so daβ es klinisch angewandt werden kann. Nach weiterer Reinigung dürfte es für die Untersuchung der Beziehungen zwischen der chemischen Struktur und biologischen Aktivität von trichophytin benutzt werden können.

## Acknowledgements

We should like to acknowledge the assistance given by Mr. A. P. Whitaker and Mr. R. Tinklin in the fermentation work and by Mr. J. H. Morris in the chemical extractions. We are also grateful for the continued interest and help of Dr. S. A. Barker, Department of Chemistry, University of Birmingham.

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# ADDENDUM

An Addendum to "THE PERFECT STATES OF *KERATINOMYCES AJELLOI* VANBREUSEGHEM, *TRICHOPHYTON TERRESTRE* DURIE & FREY and *MICROSPORUM NANUM* FUENTES".

When describing *Arthroderma uncinatum, A. quadrifidum* and *Nannizzia obtusa* in Sabouraudia **1**, 49-57, designation of the types as required by Article 35, *International Code of Botanical Nomenclature,* was inadvertently omitted. The type of *A. uncinatum* is Herb. I.M.I. 84566, the type of *A. quadrifidum* is Herb. I.M.I. 84567 and the type of *N. obtusa* is Herb. I.M.I. 84568. Valid publication of these 3 new names therefore dates from publication date of *Sabouraudia,* **1**, 2.

C. O. DAWSON & J. C. GENTLES

*Department of Bacteriology,*
*The University,*
*Glasgow, W.2.*

*Erratum* Moniliasis in Partridges, I. M. Keymer & P. K. C. Austwick, *Sabouraudia* **1**, 22-29
p. 27, line 9, for 300,000 units of Nystatin read 3,000,000 units of Nystatin

# STATUTES OF THE INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

### Article 1 NAME AND ADDRESS

The Society, founded in Paris during the VIII International Botanical Congress, 1954, is named *The International Society for Human and Animal Mycology*. The Society shall have its permanent address in Belgium.

### Article 2 OBJECTS

To encourage the practice and study of all aspects of medical and veterinary mycology.

### Article 3 MEMBERS

(1) Membership shall be open to all persons or organizations with professional or scientific interests in medical and veterinary mycology.

(2) Members may be:

(a) *Active Members*. An Active Member is one who qualifies under Article 3 (1), pays regular annual dues, and participates in the regular activities of the Society.

(b) *Patrons*. An Active Member on payment of such sum as is determined by the Assembly shall qualify as a Patron.

(c) *Emeritus Members*. An Active Member of ten years standing *and* who has reached the age of sixty-five *or* who has retired from active duty is eligible for election as an Emeritus Member.

(d) *Honorary Members*. A Member who has made extraordinary scientific contributions to medical and veterinary mycology is eligible for election as an Honorary Member. The number of Honorary Members shall not exceed three at any one time.

(e) *Sustaining Members*. An ethical corporation or organization which makes substantial annual financial contributions to the support of the Society is eligible for election as a Sustaining Member.

(3) All who join the Society during the period from its foundation in 1954 to one year after the adoption of these Statutes shall be *Charter Members*.

(4) The Members of the Society, meeting in regular business session, shall constitute the Assembly.

### Article 4 ELECTION OF MEMBERS

(1) An application for Active membership must be proposed by one Member and seconded by another Member of the Society.
Active Members shall be elected by a majority vote of Members in an Assembly or, in the interim between Assemblies, by a majority vote of the Council voting by mail.

(2) Emeritus Members shall be nominated by the Council and elected by a majority vote in an Assembly.

(3) Honorary Members shall be nominated by the Council upon receipt of a petition signed by 50 per cent of the Members and elected by a 90 per cent vote of Members voting by secret ballot at an Assembly.

(4) Sustaining Members shall be nominated by the Membership Committee and elected by a majority vote in an Assembly or in the interim between Assemblies by a majority vote of the Council voting by mail.

### Article 5 DUES

(1) The amounts of the dues for all types of membership shall be as determined by the Assembly.

(2) An Active Member, upon payment of annual dues shall be entitled to all the privileges of membership including receipt of the Journal of the Society.

(3) The Assembly shall determine the sum to be paid by an Active Member in order to qualify as a Patron. A Patron shall have all the privileges of Active membership.

(4) An Emeritus Member shall be exempted from the payment of annual dues. He shall retain all privileges of membership except that he shall receive the Journal of the Society only upon payment of a subscription equal to half the annual dues of an Active Member.

(5) An Honorary Member, who shall be exempted from the payment of annual dues, shall have all the privileges of membership.

(6) A Sustaining Member shall pay annual dues equal to or exceeding a minimum amount as determined by the Assembly. A Sustaining Member shall have all the privileges of membership except the rights to hold elective office and to vote.

(7) Payments from Patrons and Sustaining Members shall constitute an endowment fund to be used as may be decided by the Council for the support of mycological publications or for other purposes.

### Article 6 GOVERNMENT OF THE SOCIETY

The Society shall be governed by the Assembly except that between one Assembly and the next the Council shall make such decisions and take such action as may be necessary for the normal activities of the Society. Decisions of the Assembly and of the Council shall be implemented by the elected Officers and appointed Committees of the Society.

### Article 7 THE COUNCIL

(1) The Council shall consist of at least eight Officers including a President, four Vice-Presidents, a General Secretary, a Treasurer, and a Chief-Editor whose duties shall be those usually performed by such Officers. The Officers, with the exception of the Chief-Editor who shall be elected by the Council, shall be elected by the Assembly.

(2) The terms of office for all Officers shall start at the close of the Assembly at which they are elected and shall extend through the next regular Assembly of the Society. However, the term of office of the General Secretary, and of the Treasurer, may extend to a second similar term and only in special circumstances may be curtailed or extended through a

longer time by a vote of 80 per cent of the Members present at the Assembly. The Chief-Editor shall be elected by the Council at each Assembly but the same Member may be re-elected indefinitely. In order to achieve continuity, elections to the offices of General Secretary and Treasurer shall be staggered.

(3) Since Assemblies of the Society may be held at irregular intervals, if extreme irregularity leads to doubt concerning the term of Office in a particular instance, the Council may designate and define an Assembly for purpose of holding an election.

(4) Any vacancy occurring during the interim between elections may be filled by the Council and the nominee shall hold office until the next Assembly.

(5) The Council shall name an Historian to serve for an undetermined period. It shall be the duty of the Historian to accumulate and preserve facts and documents pertinent to a permanent historical record of the Society. The Historian shall not become a member of the Council by virtue of his office as Historian.

### Article 8 ELECTION OF OFFICERS

(1) In order to ensure equitable geographic distribution of the Officers of the Society, nominations for Officers other than the Chief-Editor shall be made by the Council, acting as a Nominating Committee *pro-tem*. Any Member, group of Members or official national groups may submit to the General Secretary suggestions for nominations. The Council shall consider these suggestions and shall nominate for each office two or more nominees. The names of nominees for each office shall be placed in alphabetical order, a ballot shall be mailed not less than two months before the meeting to each Member by the General Secretary, and the ballots shall be opened and counted by the Council at a meeting immediately preceding or during the Assembly. A majority vote shall elect.

### Article 9 COMMITTEES

The Council shall appoint such Standing Committees as shall from time to time be desirable. Such Committees shall act from one Assembly to the next. The incoming President shall name the Chairman of each Committee. One half of the members of any Standing Committee shall retire after each Assembly and no Member shall be eligible for immediate re-election after serving a second consecutive term. No Member shall serve on more than one Standing Committee at one time.

### Article 10 THE ASSEMBLY

(1) The Assembly has full powers to decide upon and to implement the aims of the Society.

(2) Regular Assemblies of the Society shall be held at such time and place as the Council may decide, but usually at intervals of two to five years.

(3) The Members of the Society must have brought to the attention of the Council, not less than five months before the time of an Assembly, the subjects to be discussed. The Council will add to this list such other

subjects as may seem worthy or necessary to be treated by the Assembly. The Council will send to all Members a list of the subjects to be treated not less than two months before the Assembly. On the unanimous decision of the Members in an Assembly, an Assembly may discuss and decide a subject not included in the list.

### Article 11 THE AUDITING COMMITTEE

At each regular meeting of the Society the Assembly shall appoint an Auditing Committee to audit the financial account of the Society and of its Journal. An audited statement and a summary report of income and expenses shall be published in the Journal of the Society.

### Article 12 THE JOURNAL

(1) The Society shall adopt or establish a Journal which shall serve the Society as its official organ for the publication of scientific papers and other matter of interest to medical and veterinary mycologists.

(2) The Journal shall be edited for the Society, according to the general policy laid down by the Assembly, by a Chief-Editor assisted by an Editorial Board of six or more members.

(3) The Members of the Editorial Board shall be elected by the Council and shall serve staggered terms of four years each.

### Article 13 AFFILIATED ORGANIZATIONS

(1) To facilitate co-operation between the Society and other organizations interested in medical and veterinary mycology the Council may elect such organizations to be Affiliated Societies provided that a substantial part of the members of the Affiliated Society are also members of the International Society for Human and Animal Mycology and that the Affiliated Society pays dues equivalent to one Active membership.

(2) Members of the Affiliated Societies shall be eligible to attend all meetings of the Society and will have all privileges of membership in the scientific meetings. They shall not, however, unless they are also members of the International Society for Human and Animal Mycology, hold office or vote.

### Article 14 GENERAL ARTICLES

(1) The Council shall administer all goods and funds of the Society.

(2) Decisions of the Council or of an Assembly shall be adopted by a majority vote unless some different conduct is specified in the Statutes or in the By-Laws. In case of equality the vote of the President of the Council is decisive in the Council and that of the President of the Assembly in the Assembly.

### Article 15 AMENDMENTS

These articles may be amended by a majority vote of the Members voting by a mail ballot which has been submitted by the General Secretary to the membership at the direction of the Council. Suggested amendments must have been brought to the attention of the Council of the Society in time to be sent to all Members of the Society not less than two months before an Assembly.

## BY-LAWS

### Article 1 MEMBERS

(1) Active Members shall pay an annual due of 3.00 U.S. dollars per year or the equivalent in the currency of the Member's country. In and after 1962 the annual due shall be 6.00 U.S. dollars.

(2) A Patron shall pay a single contribution of not less than 1,000 U.S. dollars or the equivalent in other currencies.

(3) Sustaining Members shall pay not less than 100 U.S. dollars per year or the equivalent in other currencies.

### Article 2 THE ASSEMBLY

The presence of 15 per cent of the Members shall constitute a quorum for the official transaction of business.

### Article 3 USE OF THE SOCIETY'S NAME

Unauthorized use of the name of the Society for advertising or other business ventures is prohibited. The circulation of any unauthorized literature shall be taken as *prima-facie* evidence of the violation of the intent and purpose expressed in this By-Law. A Member, if guilty of such violation, after being formally notified, may be expelled from the Society by a majority vote of an Assembly or of the Council.

### Article 4 SCIENTIFIC MEETINGS

(1) *Programs.* Programs of meetings shall be arranged by the Council or by a Committee elected by the Council. They shall consist of papers, lectures, symposia, films, etc.

(2) *Papers.* Members wishing to present a paper at a meeting of the Society shall present a summary and the conclusions of the paper as a concise abstract of not more than 200 words which must reach the General Secretary not less than two months before the meeting.

Members are urged not to submit titles and abstracts unless they expect to attend the meeting. Except by invitation no Member shall offer more than two papers at any one meeting, papers of joint authorship being attributed to the author reading the paper.

### Article 5 AFFILIATED SOCIETIES

In order to obtain affiliation at least one-fifth of the members of a society or organization seeking affiliation must be members of the ISHAM. The Affiliated Society should have at least 10 members, should organize at least one annual scientific meeting, should give information of its activites and a list of its members at least once every year.

### Article 6 AMENDMENTS

These By-Laws may be amended by a majority of the members voting at an Assembly provided that proposed amendments have been brought to the attention of the Council in time to be sent to all members not less than two months before the Assembly.

# OBITUARIES

## HORST UECKERT

1933-1960

Horst Ueckert was born in Eastern Germany on 2nd March 1933. He followed the career of his father, Dr. Max Ueckert, and graduated in medicine from Berlin University in January 1956. His thesis, devoted to the study of *Corynebacterium diphtheriae* clearly indicated his early interest in microbiology and not wholly satisfied with his medical studies he augmented them by a study of Botany and Zoology. In July 1959 he obtained his doctorate in the Natural Sciences with a thesis on the systematic classification of dermatophytes. After obtaining the degree of Doctor of Medicine he worked both as Scientific Assistant at the Institute of Physiology, Berlin University and in a hospital. Horst Ueckert was deeply interested in medical mycology and being qualified in both medicine and biology he well understood the basic problems involved. It was his intention eventually to try and introduce a section for medical mycology at the Robert Roth Institute in Berlin. Before his death on 5th March 1960 at the extremely early age of 27 years, Dr. Ueckert contributed six papers on the dermatophytes, two of them with his wife as co-author.

He is survived by his wife, Dr. Sonja Ueckert, who is a member of the International Society for Human and Animal Mycology, and two young children, to whom the society expresses its deepest sympathy.

R. V.

## A. VON MALLINCKRODT-HAUPT

1897-1960

Professor Asta von Mallinckrodt-Haupt was born in Bamberg, Germany on November 27th, 1897. She was educated privately and also in public schools. In 1915, after completing the Humaniora at Bamberg Gymnasium, she became a student, first at Erlangen and later in Berlin University, from which she graduated in 1922. For 10 years she was Dozent and then from 1932 until 1941, Extraordinary Professor at the Medical Academy in Dusseldorf. In 1937 she also joined the staff of the Medical Faculty of the University of Dusseldorf. From the first Asta von Mallinckrodt-Haupt was attracted by the biology of pathogenic fungi and she published many papers in this field. She was also a first class dermatologist and deeply interested in the relationship between skin symptomatology and internal diseases. Members of the society will remember her as a kindly person, and one who co-operated actively by presentation of communications to the first meeting of the society in Lisbon. The International Society for Human and Animal Mycology presents to her husband and five children its very sincere sympathy.

R. V.

## IKUYA KUBO

1908-1958

Ikuya Kubo was born in Osaka, Japan, on November 8th, 1908. After attending the High School in Kyoto, he studied Medicine at the University of Tokyo and graduated there in 1934. He remained at the Medical Clinic of the University until 1955, first as Teaching Assistant and later as Lecturer. Under the guidance of Professor Inada, Director of the Clinic and discoverer of *Spirochaeta ictero haemorrhagiae*, Dr. Kubo became interested in the spirochaetal diseases in Japan and in 1942 received the Iagkugakase degree for his studies on Autumn Leptospirosis, an epidemic disease in the River Kuyo area. His interest in medical mycology arose because of the problems of moniliasis following antibiotic therapy and because of the difficulties in the treatment of this condition. He extended his studies to the metabolism of vitamin B in connection with antibiotic therapy and to the changes induced in the intestinal flora. In 1955 Dr. Kubo became Professor of Internal Medicine at the Showa Medical College in Tokyo and shortly afterwards, in 1957, he developed the first symptoms of leukaemia. He was hospitalised, recovered for a short time, but finally succumbed to the disease on June 2nd, 1958. Professor Kubo's foremost concern at all times was for his patients of whom he had many. However, although clinical activities took up the greater part of his time, he continued to carry out research work. The society expresses to his wife and his two young sons, its very deepest sympathy.

R. V.

## N. E. GOLDSWORTHY

1897-1960

Professor Goldsworthy, who was a member of the International Society for Human and Animal Mycology since 1955, died on 26th September, 1960 after a short illness. He graduated in medicine from the University of Sydney in 1921 and later worked for a number of years in Britain, obtaining a higher degree from Cambridge University in 1928. Between 1931 and 1946 he was Senior Lecturer in Bacteriology in the University of Sydney and carried out research work on dental caries. When the Institute of Dental Research was founded after the second world war Dr. Goldsworthy became Director and he held this position until his death. In 1955 the invaluable service which he had given as secretary of the Society of Experimental Biology of New South Wales from 1932 onwards was recognized by the establishment of the Triennial Goldsworthy Lecture.

Professor Goldsworthy was the author of a great many scientific articles and he was awarded the Fairfax Reading Memorial Prize of the Dental Alumni Society of Sydney University for his outstanding contribution to dental science. He was highly respected by his colleagues and he will be missed by them both as a scientist and as a friend. To his wife and three sons the society expresses its deepest sympathy.

R. V.

# REVIEWS

*Ainsworth & Bisby's Dictionary of the Fungi*, G. C. Ainsworth (Commonwealth Mycological Institute, Kew, England, 1961) 5th Edition Pp. 547, 14 figs. 30s.

Since it was first published in 1943 there will be few who can have remained unaware of the existence of this most useful reference work and who will not welcome the appearance of the 5th revised edition. One has only to use the Dictionary to realise the comprehensive coverage of mycology which it gives and to appreciate the extraordinary amount of work entailed in its preparation and in keeping it abreast of current mycological opinion. The present edition, coming 7 years after the previous (4th) edition, contains 500 more generic names and the author believes that the list is now almost complete. The original illustrations have also been replaced and extended. As in all previous editions there are ample cross references and indications to the pertinent literature and the dictionary has also the one essential property of a good reference work, it is simple to use. To the practising mycologist the Dictionary of the Fungi is indispensable.

J. C. G.

*Animal Ringworm in Public Health: Diagnosis and Nature*. Lucille K. Georg (U.S. Department of Health, Education, and Welfare, Public Health Service, Bureau of States Services, Communicable Disease Center, 1959) 10 pl. 149 refs.

After a short general introduction the author deals briefly with the diagnosis of ringworm giving methods for collection of material and examination by microscopy and culture. In the main part of the book she deals individually with those species of dermatophytes known to cause ringworm in animals including certain of the anthropophilic species which have been found associated with animals. The text is supplemented by excellent photographic plates of typical clinical lesions on a number of animals, the microscopic characters of dermatophytes both in tissue and in culture, and the colonial form of ringworm fungi on common agar media. Well designed charts to assist diagnosis of ringworm infection are also included. The logical way in which the information is presented, the clearly written text and the helpful charts and illustrations make this a most useful book both for practising mycologists and for those in other professions who may occasionally require to investigate animal ringworm.

C. O. D.

*Fungal Diseases of Animals*. G. C. Ainsworth & P. K. C. Austwick. (Commonwealth Agricultural Bureaux, Farnham Royal, Bucks, England 1959), 1st Edition, Pp. 148. 20s.

The authors have done a commendable job in effectively summarizing for the first time in English the extensive literature on the fungal diseases of animals. This book fulfils a long-felt need since hitherto much of the information presented could only be obtained through laborious library research.

The book is divided into seventeen chapters of which 15 are devoted to the infectious mycoses and 2 to the mycotoxicoses of animals. The chapters are well organized and a wealth of information is presented in a clear concise manner. Supplementing the text are eight black and white and four colour plates of the finest quality. The book also contains an excellent index and a valuable bibliography culled from the past as well as the recent world literature.

This book was not intended by the authors to serve as a comprehensive text on veterinary mycology. The techniques employed in establishing a laboratory diagnosis of a mycotic disease are not given in detail. Likewise, the cultural characteristics of a number of the etiologic agents are briefly described and are not supplemented by adequate illustrations. Therefore, students of veterinary mycology will have need of additional sources of information.

The authors have succeeded admirably in preparing a concise, well-written review of fungal diseases of animals and the book is to be regarded as a valuable contribution to veterinary medicine and to medical mycology as well. This reviewer can heartily recommend this publication as a valuable reference to anyone interested in the mycoses of animals.

W. K.

*Practical Mycology*: *Manual for Identification of Fungi*. SIGURD FUNDER. (A. W. Brøggers Boktrykkeri A/S, Oslo, Norway, 1961) 2nd Edition Pp. 123. $6.50.

This second edition varies only slightly from the previous one, the text being brief and merely supplementary to a series of excellent diagrams. All the diagrams pertaining to medical mycology and a large number of the others are the work of J. Walter Wilson & Orda A. Plunkett (*Practical Medical Mycology*) to whom the book is dedicated. There is also a useful little glossary and "A Key to the Human Mycoses" by Morris A. Gordon (*J. Bact.* (1952) **63**, 385-392) is included. The introductory sections dealing with the fundamentals of mycology and classification of the fungi are rather brief but they are nevertheless clear, logical and easily understood, as is the treatment of laboratory methods. The manual, which is well bound, attractive in appearance and printed on paper of excellent quality will be a useful addition to laboratories where fungi are occasionally encountered and where the services of a trained mycologist are not readily available. It is to fill this need that it was designed.

J.C.G.

### *Bulletin de la Société Francaise de Mycologie Médicale*

The first 2 numbers of this bulletin, which will appear at intervals in the future, have recently been published. Number 1 gives the proceedings of a meeting of the society held on 8th April 1960 at which papers dealing with new aspects of therapy of the mycoses were presented and discussed. Eight papers reporting the use of griseofulvin, one on the use of amphotericin B and one on *in vitro* studies of both drugs are given. Bulletin number 2 contains 5 papers on varied topics which were presented at a meeting held on 13th January 1961 and a notice of a film "Teignes et thérapeutiques modernes" which was also shown. Both issues contain book reviews and in number 2 there is also a news section in which *Sabouraudia* is reviewed and the next meeting of the society in June 1961 is intimated. In a foreword Dr. Rivalier explains that the decision to produce the bulletin was made in order to permit those members living some distance from Paris, where the meetings are usually held, to follow the activities of the society. Such a step was justified by the rapid widening of the scope of medical mycology and the ever increasing interest which is being taken in the subject. The bulletins, which are published by the Service de Mycologie, Institut Pasteur, are small (approximately 15 pages) and not illustrated. They will nevertheless suit the purpose for which they are designed and the Société Francaise de Mycologie Médicale have set an example which other national societies might well follow.

J. C. G.

### *The Japanese Journal of Medical Mycology*

The first volume of the Japanese Journal of Medical Mycology was published in 1960. The volume is made up of 4 parts each approximately 100 pages in length and contains many original articles although some review articles are also included. As all but 3 articles are in Japanese it would seem that the main object of the publication is to ensure diffusion of information throughout Japan and this the journal will surely do. The paper used for the text of the articles is not of particularly good quality but the illustrations which are of a high standard throughout are printed on an excellent smooth paper. The Japanese Society for Medical Mycology are to be congratulated on their initiative. The society has approximately 500 members and 39 of them are also members of ISHAM.

R. V.

## FILMS

### *Fungus Infection Treated with Griseofulvin*
### *Veterinary Experience with Griseofulvin*

[Produced by Glaxo Laboratories Ltd., Greenford, Middlesex, England]

These two films are supplementary to *The Griseofulvin Story* (*Sabouraudia* 1, 69). The first, which is of interest particularly to dermatologists and general medical practitioners, consists of clinical demonstrations of 6 cases before and after treatment. These are infections of the scalp (2), the body (2) and the beard (1). The sixth case, which is of a nail infection, is remarkable and shows the complete regrowth of a thumb nail which had been infected down to the nail bed with *Trichophyton rubrum.* This involved the patient attending a hospital to have the nail photographed twice a day, every day for 5½ months. The time taken in the film to show the regrowth of the nail is 33 seconds.

The veterinary film gives details of the action of griseofulvin on fungi and illustrates the way in which dermatophytes are eradicated from the body during treatment with oral griseofulvin. Clinical demonstrations of ringworm infections in various domestic animals before, during and after treatment are presented in an excellent manner. Both films are in colour, 16 mm., with a sound commentary in English and are available on loan by application to the Public Relations Service, Glaxo Laboratories Ltd. The first runs for 12 minutes, the second for 15 minutes.

## SCIENTIFIC MEETING AND FOUNDATION OF A GERMAN SPEAKING BRANCH OF ISHAM

### *Subject of discussion: Griseofulvin in dermatology*

On 15th January 1961 the first scientific meeting of the German speaking members of ISHAM took place in Essen. By invitation of Professor Götz a German speaking branch of ISHAM was constituted in order to promote the personal and scientific contacts among mycologists of the German language. In the presence of the general secretary of ISHAM, Professor Vanbreuseghem, the participants, numbering about 100, elected the executive committee: Professor Götz, Essen (chairman), Dr. Rieth, Hamburg (secretary), Dr. Janke, Fulda (treasurer). Professor Memmesheimer, Essen, the present chairman of the German Dermatological Society, and Professor Grütz, Bonn, were elected as honorary members. Contact with other mycological societies will be undertaken by Dr. Seeliger, Bonn.

In the name of Professor Mackinnon, President of ISHAM, Professor Vanbreuseghem, Antwerp, delivered the greetings of the executive committee of ISHAM. Professor Franquet, Nancy, vice president of the French Medical Mycological Society, attended as a guest and a particular welcome was accorded Dr. Scholer, Basel, Professor Braun, Magdeburg, and Professor Windisch, Berlin.

After an interesting review on the development of medical mycology and the beginning of ISHAM by Professor Vanbreuseghem, 18 papers were read under the topic "Griseofulvin in Dermatology". Those were followed by extended and lively discussions.

CONTRIBUTED.

## SOCIETY NEWS

It is understood that invitations have already been sent to the speakers for the symposium on "The Influence of the Environment on the Epidemiology of Mycoses" which is to be held at the 8th International Congress of Microbiology, Montreal. An assembly of ISHAM will be held either immediately before or after the Congress and members will be notified of the exact date as soon as possible. Any member wishing to raise society business or to present a scientific paper should approach the secretary as prescribed by the statutes and bye-laws. Members must make their own arrangements for travel and accommodation and European members are reminded that as the Congress takes place in the summer season early reservations are necessary.

It is intended that a list of Members should be published in *Sabouraudia* in the near future. In order that this list will be accurate Members are asked to check carefully the address which has been used to forward this journal and to notify the editor of any inaccuracies as soon as possible.

Members submitting papers for publication are requested to ensure that they are written in the correct format. The arrangement of headings and sub-headings, explanations of plates and figures, references to literature and other details should be clear from the information given in "Directions to Contributors" and by reference to this and the previous issue of the journal.

## INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

### Income and Expenditure Account

*For the year ending 31/12/60*

| RECEIPTS | | PAYMENTS | | |
|---|---|---|---|---|
| Balance in hand at 1st January 1960 | 321,943 | Administrative costs | | 23,940 |
| Subscriptions | 38,556 | Stationery | | 1,297 |
| Bank interest | 1,303 | Subscriptions to F.A.I.B. and U.A.I. | | 410 |
| | | Editor's expenses | | 5,025 |
| | | Bank charges | | 230 |
| | | Balance at 31st December 1960 | | |
| | | in Bank of Antwerp | 37,424 | |
| | | in Midland Bank, London | 293,476 | |
| | | | | 330,900 |
| Belgian Francs | 361,802 | Belgian Francs | | 361,802 |

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# THE APPLICATION OF FLUORESCENT ANTIBODY TECHNIQUES TO MEDICAL MYCOLOGY—A REVIEW

W. Kaplan and L. Kaufman

*Department of Health, Education and Welfare, Public Health Service,*
*Communicable Disease Center, Laboratory Branch,*
*Atlanta 22, Georgia.*

A review of the significant applications of fluorescent antibody (FA) techniques in medical mycology is presented. The review covers the progress made in the development of FA reagents for the detection of *Cryptococcus neoformans, Candida albicans,* and other *Candida* spp., *Sporotrichum schenckii, Histoplasma capsulatum,* and *Blastomyces dermatitidis.* The progress made in the application of this technique to measurement of fungal antibodies in sera is described

The authors briefly discuss the basic principles of the FA procedure, some of the problems encountered in performing the tests and the potentialities of the technique.

With the advent of the Coons (Coons, Creech, Jones & Berliner, 1942) fluorescent antibody (FA) technique a new serological tool became available for the rapid detection and identification of micro-organisms. Basically the FA technique is an immunological staining procedure which allows the microscopic visualization of an antigen-antibody reaction. Such visualization is accomplished through the use of antibody coupled to a fluorochrome. The fluor-antibody complex is often referred to as the labelled antibody or conjugate. The union of the labelled antibody and its homologous antigen results in a product that fluoresces when excited by light of the proper wave length. To observe this fluorescence one uses a fluorescence microscope. This consists of a standard microscope fitted with a cardioid darkfield condenser. Additional requirements are a very bright light source which yields a powerful flow of energy particularly in the short wave length region of the spectrum and selective filters for control of the fluorescence excitation and emission wave lengths.

The fluorochromes most frequently used to date have been derivatives of fluorescein, a dye which fluoresces with a yellow-green color. Coons and his associates (1942) and other early workers (Goldman & Carver, 1957) used fluorescein isocyanate as a labelling compound. However, this derivative was unstable in the presence of moisture and thus presented certain practical difficulties. This disadvantage was overcome by Riggs and co-workers. (Riggs, Seiwald, Burkhalter, Downs & Metcalf, 1958) who synthesized a more stable fluorescein derivative, fluorescein isothiocyanate. This fluorochrome is at present the most widely used labelling agent. Fluorochromes of other colors, such as rhodamine and its derivatives, which fluoresce with a reddish color, have also been employed as labelling compounds.

The simplest FA procedure involves the application of solutions of labelled antibody directly on dried smears of antigen(s). This technique for the detection of antigens is termed the direct method. One commonly employed modification of this basic procedure is the indirect method. Here the antigen-antibody reaction is made visible by the addition of a conjugate directed against globulin of the animal producing the initial antibody. For example, the reaction of *Histoplasma capsulatum* antibodies in human serum with homologous antigen may be made evident by treatment of the complex with labelled rabbit anti-human gamma globulin antibodies. The indirect method can be employed as a tool for the rapid detection of antibodies or for the identification of antigens. An extensive description and discussion of FA methods may be found in the manual prepared by Cherry and his associates (Cherry, Goldman & Carski, 1960) and in the review of Beutner (1961).

The FA technique has several important advantages over conventional diagnostic methods. First, fluorescing antigen-bearing cells with distinct morphological characteristics can be observed. Second, fluorescent cells even when few in number or in mixture with other organisms can easily be detected against a dark background. Antigen-bearing organisms need not be viable for detection. Another advantage of the FA procedure is its rapidity. The time required for carrying out conventional isolation, purification and identification procedures is eliminated.

Subsequent to the introduction of the technique by Coons *et al.* (1942) microbiologists applied the technique to the rapid diagnosis of bacterial (Thomason, Moody & Goldman, 1956), viral (Goldwasser & Kissling, 1958), and protozoan diseases (Goldman, 1954). In general, the results have been extremely satisfactory and in some systems have had practical application. A review of the literature discloses that concurrent studies in the area of medical mycology have lagged behind those carried out in other fields of microbiology. This apparent neglect was due in part to the discouraging observation by earlier workers that hyphae and spores of some fungi were autofluorescent. Unfortunately, the filters used by these early workers were unsuitable and, as a result, the fungal elements of some species autofluoresced with a greenish color simulating that of fluorescein. This made it difficult to differentiate between autofluorescent and fluorescein-stained cells. Subsequently, it was found that this problem could be eliminated through the use of proper filters. It is the authors' experience that a combination of a 5113 Corning glass primary filter, 3 mm. in thickness, and a Wratten 2A secondary filter, 2 mm. in thickness, is satisfactory. Further studies may show that other filter combinations are superior.

Within the past 3 years, a definite increase in interest in determining the applicability of FA techniques to medical mycology developed. Many investigators have initiated studies designed to evaluate this immunochemical staining procedure as a tool for the rapid detection of fungi or fungal antibodies. While to date these investigations have been preliminary in nature, the findings have been uniformly favourable.

This review was undertaken for the purpose of acquainting the microbiologist with the significant FA contributions made to date in medical mycology.

## IMMUNOFLUORESCENT STAINING OF FUNGI

### *Cryptococcus neoformans*

Among the early reports on the application of the FA technique in medical mycology is that by Eveland and his associates (Eveland, Marshall, Silverstein, Johnson, Iverson, & Winslow, 1957). They employed the technique in a study of the distribution of *C. neoformans* and its polysaccharide breakdown products in formalin-fixed tissues. The FA procedure permitted these workers to observe the yeast and its polysaccharide antigens in the tissues.

More recently, Kase & Marshall (1960) used the FA technique for the identification of *C. neoformans* in culture. Of 92 isolates of this fungus tested, 91 were stained with labelled rabbit anti-globulins prepared against strains of *C. neoformans* identified as Evans A, Neill 1 and Neill 5. The isolate which did not react belonged to Evans' *C. neoformans* serotype C (Evans, 1950). Kase & Marshall, however, were able to stain this lone strain with conjugates produced from antiglobulins against either Evans' *C. neoformans* serotype B or C. Although the conjugates used in these studies stained the capsular material, the intensity of the stain was not affected by capsule size. These workers further reported that their conjugates stained 4 isolates of *C. neoformans* var. *innocuous*, which is considered by many workers to be a distinct species, *C. diffluens*. However, no other cross-reactions were noted when their conjugates were applied to smears of 23 species of heterologous yeasts belonging to genera other than *Cryptococcus*. None of the cryptococci studied reacted with conjugates prepared from antisera for *Torulopsis glabrata, Saccharomyces cerevisiae, Histoplasma capsulatum, Blastomyces dermatitidis,* and *Coccidioides immitis.*

The aforementioned studies though contributory are essentially preliminary in nature. Additional work must be done to further rule out cross-reactions of *C. neoformans* conjugates with heterologous species of *Cryptococcus* and other genera before such reagents can be employed for the unequivocal identification of *C. neoformans.*

### *Candida albicans and other Candida species*

Preliminary studies have also been carried out on the application of the FA technique to the identification of members of the genus *Candida.* Gordon (1958a) described in detail the production and use of 2 fluorescein isocyanate labelled rabbit anti- *C. albicans* globulins. Both conjugates stained cells of *C. albicans* in clinical materials and cultures. However, 1 of Gordon's conjugates in addition reacted not only with the cells of *C. tropicalis, C. stellatoidea, C. parakrusei, C. krusei, C. pseudotropicalis,* and *C. guilliermondii,* but also with the cells of 5 species of heterologous yeasts representing genera other than *Candida.* Adsorption of this labelled antiglobulin with cells of *C. parakrusei* eliminated all of the aforementioned cross-reactions except that with *C. tropicalis* and some strains of *C. stellatoidea.* Gordon's other conjugate stained both *C. albicans* and *C. tropicalis,* but it also showed little or no reaction with other *Candida sp.*

In a second report, Gordon (1958b) stated that it was possible to differentiate *C. albicans* from *C. stellatoidea* with fluorescent antibody. Differentiation was accomplished by employing labelled anti- *C. albicans* globulins that had previously been adsorbed with cells of *C. parakrusei*. Adsorption of the conjugate with cells of *C. stellatoidea* apparently eliminated too much of the reactivity for *C. albicans*. This *C. parakrusei* adsorbed conjugate stained cells of 60 isolates of *C. albicans* at an intensity rated as 3 to 4+ and 2 isolates at an intensity of 2+ However, this adsorbed conjugate also stained the cells of 3 isolates of *C. stellatoidea* with a 1+ intensity, 3 with a 2+ intensity and 1 with a 3+ intensity. A second conjugate was reported not to require any antibody adsorption to render it specific. It stained cells of 13 isolates of *C. albicans* at a 4+ level. In contrast, 6 isolates of *C. stellatoidea* reacted with a 0 to 1+ intensity and 1 isolate with a 2+ intensity. Similar staining reactions were obtained with a conjugate prepared from *C. tropicalis* rabbit antiglobulins.

This study revealed the extent to which common antigens are found among the species of *Candida*. Furthermore, the variable staining intensities encountered with the strains of *C. stellatoidea* emphasize the need for more specific reagents for precise identification of the species.

The present authors have also initiated studies designed to apply the FA technique to the rapid identification of *C. albicans* and other *Candida* species. Although these studies are still in progress, several interesting discoveries have been made. Fluorescein isothiocyanate labelled rabbit anti- *C. albicans* globulins adsorbed with cells of *C. stellatoidea* revealed the existence of 2 distinct serological groups of *C. albicans*. One group is serologically similar to, if not identical to, *C. stellatoidea*; the other group is serologically similar to *C. tropicalis*. These observations revealed through the use of the FA technique are in accord with the findings of Hasenclever & Mitchell (1961) who reported the existence of 2 such groups of *C. albicans* on the basis of agglutination tests. Kaplan & Kaufman (1961) have also observed that it is possible to differentiate *C. tropicalis* from *C. albicans* and *C. stellatoidea* through the use of fluorescein isothiocyanate labelled *C. albicans* antibodies that had been adsorbed with cells of *C. tropicalis*.

Several investigators have studied the applicability of using FA reagents for the rapid detection of *C. albicans* in clinical materials. Kunz (1958a) investigated the relationship of *C. albicans* to pneumocysts of interstitial plasma cell pneumonia. Using fluorescein isocyanate labelled rabbit anti- *C. albicans* globulins, this worker demonstrated the presence of *C. albicans* in the lungs of 2 of 3 premature infants who had died of interstitial plasma cell pneumonia. In no case did he observe a reaction between conjugate and pneumocyst. In another study, Kunz (1958b) successfully demonstrated the capacity of his conjugate to detect *C. albicans* in tissues of experimentally infected mice.

Kemp & Solotorovsky (1960) through the use of lissamine rhodamine B 200 and fluorescein isothiocyanate labelled rabbit anti- *C. albicans* globulins demonstrated the presence of the organism in frozen kidney tissue sections of experimentally infected mice.

### *Sporotrichum schenckii*

Kunz (1959) labelled rabbit anti- *S. schenckii* globulins with fluorescein isocyanate and successfully stained the cells of this fungus in culture, in frozen sections, and smears from experimentally infected animals. None of 18 heterologous species tested by this worker reacted with the labelled antibody solutions.

Kaplan & Ivens (1960) also carried out studies on the application of the FA technique to the detection of *S. schenckii* in cultures and clinical materials. Fluorescein isothiocyanate labelled rabbit anti- *S. schenckii* globulins were shown to stain both the yeast and mycelial phase of the fungus. Conjugates diluted 1:4 to 1:12, depending upon the lot tested, showed bright staining of the fungus in routine tests. Of 47 strains of 21 heterologous fungous species representing 12 different genera studied, none were stained by the prepared conjugates. These workers also demonstrated the presence of *S. schenckii* cells in smears prepared from infected mouse testes. Furthermore, their conjugates also stained the cells of this fungus in formalinized tissue sections of an experimentally infected mouse and rat.

Kaplan & Gonzales Ochoa (1960) using labelled *S. schenckii* antiglobulins revealed this fungus in smears prepared directly from lesion exudates of 10 out of 12 culturally confirmed human cases of sporotrichosis. The immunofluorescent staining technique enabled these workers to find fungous elements in smears from a human skin lesion which was negative for *S. schenckii* by conventional cultural procedures. This patient, however, did give a positive skin test reaction with sporotrichin. These results indicate the sensitivity of the FA reagents and exemplify the advantage of these reagents for the rapid diagnosis of a disease where few or non-viable cells are present in clinical materials.

*Histoplasma capsulatum*

Gordon (1959) stained the yeast phase cells of five strains of *H. capsulatum* with fluorescein isocyanate labelled anti- *H. capsulatum* globulins. This conjugate also stained the small buds of *H. duboisii*, and the buds of an aberrant strain of *H. capsulatum.* In addition, this conjugate showed variable staining intensities upon reaction with 4 strains of *Blastomyces dermatitidis*. Gordon attempted to eliminate these cross-reactions by adsorption of the conjugate with cells of *B. dermatitidis*. His attempt resulted in the conjugate's loss of staining reactivity for both the homologous and heterologous organisms. However, Gordon did find that dilution of the non-adsorbed conjugate 1:4 resulted in a reagent which showed 3 to 4+ staining of *H. capsulatum* yeast cells, the buds of *H. duboisii* and *Paracoccidioides brasiliensis,* and the cell walls of some strains of *B. dermatitidis*. Two protozoan species, *Toxoplasma gondii* and *Leishmania donovani* did not react with this reagent. In addition, 16 strains of 7 heterologous species representing 6 genera were not stained by his diluted conjugate.

Although judicious use of such a diluted reagent might allow differentiation of *H. capsulatum* from heterologous fungi, a species specific conjugate would be preferable.

More recently, Kaufman & Kaplan (1961) conjugated rabbit anti- *H. capsulatum* globulins with fluorescein isothiocyanate. Their labelled antiglobulins, in addition to staining 6 strains of *H. capsulatum* exhibited strong cross-staining with up to 28 fungus strains representing 18 heterologous species of 11 genera. The cross-staining exhibited by these conjugates serve to illustrate the widespread existence of common antigens among the various genera of fungi.

Adsorption of the labelled antibodies either twice with cells of *B. dermatitidis* or twice with cells of *Coccidioides immitis*, and once with cells of *B. dermatitidis* resulted in a FA reagent, specific for yeast phase cells of *H. capsulatum*. In limited studies, these workers have shown that the specific conjugates could be used to readily detect *H. capsulatum* cells in tissue smears made from exudates and organs of experimentally infected mice.

The possibility of employing the FA procedure in epidemiological studies was investigated by Kaplan and associates (Kaplan, Ajello, Di Bitetto, & McDonough, 1961). They applied the method to the rapid detection of *H. capsulatum* in soils using unadsorbed fluorescein isothiocyanate labelled rabbit anti-*H. capsulatum* globulin to stain smears prepared from saline suspensions of soil samples. Their study of 6 soil samples, shown by conventional techniques to contain viable elements of *H. capsulatum*, revealed the presence of several highly fluorescent round to oval forms. The morphology of these forms was consistent with that of the microconidia of *H. capsulatum*. The characteristic tuberculate spores of this fungus were not detected in the soil samples. It is obvious that additional studies are necessary to evaluate the reliability and practicality of this technique for the detection of *H. capsulatum* in soil specimens.

### *Blastomyces dermatitidis*

Although FA reagents for *B. dermatitidis* have been produced in various laboratories, none have as yet achieved a desirable degree of specificity. Gordon (1958a) labelled rabbit anti- *B. dermatitidis* globulins with fluorescein isocyanate. His conjugate stained yeast cells of *B. dermatitidis* and *C. albicans*, and showed little or no cross-reaction with *P. brasiliensis* or *H. capsulatum*.

Kaplan & Kaufman (1961) have labelled rabbit anti- *B. dermatitidis* globulins with fluorescein isothiocyanate. Their preparations brightly stained the yeast and mycelial phases of *B. dermatitidis* and *H. capsulatum*. These reagents also stained the cells of *B. dermatitidis* present in smears of lesion exudates obtained from experimentally infected mice and a naturally infected dog.

## Detection of Antibody by Immunofluorescent Staining

Vogel & Padula (1958) carried out a preliminary study of the adaptability of the indirect FA method for measurement of fungal antibodies in human sera. For the second stage reaction they used fluorescein isocyanate labelled rabbit anti-human globulins. Serum samples from 1 patient with cryptococcal meningitis were tested for antibody activity. One undiluted early sample resulted in the *C. neoformans*-antiglobulin complex being stained brightly. Diminished staining was observed when this sample was applied at a 1:2 dilution. No reaction was detected when the serum was diluted 1:4. Slight staining of the antigen was observed with serum collected 4 months later from this patient. Slight staining of the antigen was also observed when sera from apparently normal individuals were employed. However, adsorption of these latter sera with cells of *C. albicans* eliminated the weak reactions. Elimination of staining was not observed when the patient's sera were adsorbed with *C. albicans*.

Vogel & Padula also tested 3 other sera from patients with clinical manifestations of mycotic disease. One of these sera had a complement-fixation (CF) titer of 1:256 with *B. dermatitidis* antigen, the second gave a CF titer of 1:128 with *H. capsulatum* antigen and the third agglutinated *C. albicans* antigen at a dilution of 1:256. These serum samples gave identical titers with the indirect FA method. Their study indicated the potentialities of the FA procedure for the rapid detection and measurement of fungal antibodies.

## Discussion

The results of the preliminary studies reported to date are most encouraging and point to the potential value of the FA technique for the rapid diagnosis of fungal diseases. However, much remains to be done before this immunochemical staining procedure can routinely be employed in the diagnostic laboratory. Carefully planned and controlled studies must be carried out to produce species specific conjugates. The extensive spread of common antigens among the fungi makes the utilization of such reagents mandatory. Furthermore, the reliability of results obtained through use of such reagents should be carefully evaluated by conventional cultural and biochemical tests.

The production of specific conjugates by current procedures inadvertently results in the loss of specific antibody. Since the sensitivity of specific labelled antibody is related to concentration, it is important to utilize antigen preparations and immunization schedules which best elicit maximum antibody production in the host animal. In the field of medical mycology additional basic investigations along these lines have to be carried out.

Discrepancies in results obtained by investigators in different laboratories frequently can be explained on the basis of variation in the immunizing strain, immunization schedule, the flurochrome used in labelling, host reactivity, antibody titers, and the optical equipment used. Before FA procedures can be routinely employed in diagnostic laboratories, standardization of reagents, equipment and operational procedures should be established so as to make results comparable.

In carrying out FA tests for the diagnosis of fungal diseases, it is imperative that adequate controls be employed. In all cases, labelled normal serum globulins should be employed along with labelled immune globulins. This practice would serve as a safeguard against the possibility that the staining reactions observed were cause by "normal" antibodies, non-specific reactions, or by innate fluorescence.

Frequently individual cells are noted in a preparation that fluoresce with an abnormal color. To confirm whether or not such fluorescence is innate, the authors have found it desirable to examine unstained preparations under the fluorescence microscope.

## Zusammenfassung

In einer Übersicht werden die bedeutsamen Anwendungsgebiete der Verwendung fluoreszierender Antikörper in der medizinischen Mykologie geschildert. Das Referat umfaβt die Fortschritte, die in der Entwicklung der fluoreszierenden Antikörper-Technik zum Nachweis von Cryptococcus neoformans, Candida spp. sowie von Sporotrichum schenckii, Histoplasma capsulatum und Blastomyces dermatitidis erzielt wurden. Ferner werden die mittels dieser Technik gemachten Fortschritte bei der quantitativen Serumantikörperbestimmung beschrieben. Die Verff. erörtern kurz die Grundlage des Verfahrens, einige der aufgetretenen Probleme und die potentiellen Möglichkeiten.

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# SEROLOGICAL CLASSIFICATION OF THE GENUS *TORULOPSIS*

T. Tsuchiya, Y. Fukazawa and S. Kawakita

*Department of Bacteriology, School of Medicine, Juntendo University, Tokyo.*

Antigenic analyses of 4 species of *Torulopsis* were carried out by the slide agglutination method using monospecific and absorbed antisera homologous to antigens of the genus *Candida*. The antigenic structure of each species was confirmed by reciprocal or successive absorption and slide agglutination test. *Torulopsis stellata* var. *cambresieri* and *T. colliculosa* possess the thermostable antigens 1, 3, 4 and 24, but no thermolabile antigen. Their antigenic structures were identical to those of *Saccharomyces rosei* and *S. fermentati*, respectively, despite the biological differences among them. *Torulopsis holmii* has thermostable antigens 1, 3, 4, 5, 6, 10 and 26, but no thermolabile antigen. The antigenic structure of this species is similar to that of *S. exiguus*. *Torulopsis glabrata* contains the thermostable antigens 1, 3, 6, 10 and 34, and thermolabile antigen *k*. In a comparative study, the antigenic structures of many strains coincided with those of the standard when they were analyzed with monospecific antisera. Consequently, the absorbed or monospecific antisera can be used for rapid species identification, and the antigenic structures of various species can be used in their classification.

Strains of the genus *Torulopsis* are not infrequently isolated from human sources. Rieth (1958) isolated many strains of *Torulopsis famata, T. glabrata* and *T. holmii,* mainly from the patients with skin diseases. Mizuno & Tsuji (1959) reported that the number of strains of *T. glabrata* isolated from patients suffering from endovaginitis was second only to *Candida albicans.* However, several investigators have shown that *T. glabrata* is not a pathogen. On the other hand, Black & Fisher (1937) and Mizuno & Tsuji (1959) state that this organism may possess some pathogenicity for mice and rats. Wickerham (1957) identified 2 strains of *T. glabrata* from blood cultures.

Berlese (1895) created the genus *Torulopsis*, and Lodder & Kreger-van Rij (1952) amended the designation. The genus *Torulopsis* includes 22 species and 1 variety. Before amendment, the genus *Cryptococcus* included many of the species of the genus *Torulopsis*. According to the classification of the subfamily *Cryptococcoideae* by Lodder & Kreger-van Rij, each of the genera *Torulopsis*, *Candida* and *Cryptococcus* manifests biologically related characteristics but different properties. The genera *Torulopsis* and *Candida* differ from the genus *Cryptococcus* in that they lack the ability to produce starch and in the fermentative properties with several sugars. The genus *Torulopsis* differs from the genus *Candida* by the absence of pseudomycelium formation or in the very primitive pseudomycelium formation.

Benham (1935) showed that *C. albicans* and *T. glabrata* possessed common antigens. Seeliger (1958) indicated that serological relationships existed among *T. glabrata* and *T. inconspicua* and several asco- and anascosporogenous yeasts when tested by agglutination and precipitin reactions. Recently, Hasenclever & Mitchell (1960) studied the antigenic relationships of *T. glabrata* and 7 species of the genus *Candida,* and demonstrated that *T. glabrata* had a specific antigen. We have also observed in the course of analyzing antigens of many ascosporogenous and anascosporogenous yeasts that *Candida* antiserum agglutinated some strains of the genus *Torulopsis*. This paper is concerned with the experimental results obtained from antigenic analyses of *T. stellata* var. *cambresieri*, *T. colliculosa*, *T. holmii* and *T. glabrata*, in relation to the antigens of the genus *Candida*.

## MATERIALS AND METHODS

*Strains of yeast used*: *Torulopsis stellata* var. *cambresieri* (IFO 0704), *T. colliculosa* (IFO 0381), *T. holmii* (IFO 0660) and *T. glabrata* (IFO 0005) were obtained by courtesy of Dr. Hasegawa of the Institute for Fermentation in Osaka. Many of the strains used for antigenic analyses in previous investigations were used again for the preparation of monospecific antisera homologous to the established antigens.

The methods for preparing antigens, the immunization of rabbits, the absorption of agglutinins and slide agglutination tests, as well as the method of immunization of animals and the procedures for preparing absorbed antisera have been described previously (Tsuchiya, Fukazawa, Hayashi, Hayashi & Doi, 1957). The biological characteristics of the various strains employed were examined mainly by the method described by Lodder & Kreger-van Rij (1952).

## RESULTS

*Thermostable antigens*: The study was concerned with the detection of thermostable and thermolabile antigens. For antigenic analyses, slide agglutination tests were first carried out using monospecific or absorbed antisera homologous to antigens of the genus *Candida* as previously described (Tsuchiya, Fukazawa, Sano, Shimura & Murata, 1960). These antisera were used because cross

| *Monospecific or absorbed antiserum* | | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Heated cells used as antigens for agglutination | T. st. | + | + | + | − | − | − | − | − | − | − | − | − | − |
| | T. col. | + | + | + | − | − | − | − | − | − | − | − | − | − |
| | T. hol. | + | + | + | + | + | − | − | − | + | − | − | − | − |
| | T. glb. | + | + | + | − | + | − | − | − | + | − | − | − | − |
| *Monospecific or absorbed antiserum* | | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
| Heated cells used as antigens for agglutination | T. st. | − | − | − | − | − | − | − | − | − | + | − | − | − |
| | T. col. | − | − | − | − | − | − | − | − | − | + | − | − | − |
| | T. hol. | − | − | − | − | − | − | − | − | − | − | − | + | − |
| | T. glb. | − | − | − | − | − | − | − | − | − | − | − | − | − |

T. st., *T. stellata* var. *cambresieri*; T. col., *T. colliculosa*; T. hol., *T. holmii*; T. glb., *T. glabrata*.

TABLE 1. RESULTS OF SLIDE AGGLUTINATION TESTS OF FOUR SPECIES OF THE GENUS *Torulopsis* AGAINST MONOSPECIFIC OR ABSORBED ANTISERA HOMOLOGOUS TO THERMOSTABLE ANTIGENS OF THE GENUS *Candida* (EXPERIMENT A).

reactions existed among species of the genera *Torulopsis* and *Candida*. Monospecific antisera homologous to every antigen may be prepared in several ways, depending on the species employed for immunization and for absorption. In the present study, however, the combination of species employed for the preparation of monospecific antisera were similar to those described in previous reports (Tsuchiya *et al.*, 1957; Tsuchiya, Fukazawa & Kawakita, 1959).

In Experiment A, as shown in table 1, 26 different monospecific or absorbed antisera, homologous to thermostable antigens of the genera *Candida, Saccharomyces* and *Hansenula,* were used for the slide agglutination test. *Torulopsis stellata* var. *cambresieri* and *T. colliculosa* showed positive agglutination reactions against monospecific or absorbed antisera homologous to antigens 2, 3, 4 and 24. Also, *T. holmii* showed positive agglutination reactions against monospecific or absorbed antisera homologous to antigens 2, 3, 4, 5, 6, 10 and 26, and *T. glabrata* was positive against the sera homologous to antigens 2, 3, 4, 6 and 10. Therefore, it can be concluded that *T. stellata* var. *cambresieri* and *T. colliculosa,* have the thermostable antigens 2, 3, 4 and 24, and that *T. holmii* has the thermostable antigens 2, 3, 4, 5, 6, 10 and 26 with respect to the antigens previously studied (Tsuchiya *et al.*, 1960). Similarly, *T. glabrata* demonstrated the possession of thermostable antigens 2, 3, 4, 6 and 10.

A subsequent study (Experiment B) was carried out to find a thermostable antigen in addition to those reported earlier. Table 2 shows, for example, that in experiment 8 heated *T. glabrata* antiserum absorbed with heated cells of *C. albicans* showed positive agglutination reactions against heated cells of *T. glabrata* as well as with *T. holmii,* 2 species of the genus *Candida* and 2 species of the genus *Saccharomyces* (table 2). These reactions were interpreted on the basis

| *Exper. No.* | *Antiserum of heated cells or antiserum absorbed in Exp. (No.)* | *Heated cells for absorption* | *Heated cells used as antigens for agglutination* | | | | | | | | | | | | | | | *Thermostable antigens homologous to antibodies remaining after absorption* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *T. st* | *T. col.* | *T. hol.* | *T. glb.* | *C. alb* | *C. pt.* | *C. rob.* | *C. kr.* | *C. pk.* | *C. glm.* | *C. pel.* | *S. bis.* | *S. ros.* | *S. exg.* | *S. fer.* | |
| 1 | T. st. | C. alb. | + | + | − | − | − | − | − | − | − | − | − | − | + | − | + | 24 |
| 2 | As(1) | S. ros. | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | |
| 3 | T. col. | C. alb. | + | + | − | − | − | − | − | − | − | − | − | − | + | − | + | 24 |
| 4 | As(3) | S. ros. | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | |
| 5 | T. hol. | C. alb. | − | − | + | + | − | + | + | − | − | − | − | + | − | + | − | 10, 26 |
| 6 | As(5) | C. rob. | − | − | + | − | − | − | − | − | − | − | − | − | − | + | − | 26 |
| 7 | As(6) | S. exg. | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | |
| 8 | T. glb. | C. alb. | − | − | + | + | − | + | + | − | − | − | − | + | − | + | − | 10, 34 |
| 9 | As(8) | C. rob. | − | − | − | + | − | − | − | − | − | − | − | − | − | − | − | 34* |
| 10 | As(9) | T. glb. | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | |

C. alb., *C. albicans*; C. pt., *C. pseudotropicalis*; C. rob., *C. robusta*; C. kr., *C. krusei*; C. pk., *C. parakrusei*; C. glm., *C. guilliermondii*; C. pel., *C. pelliculosa*; S. bis., *S. bisporus*; S. ros., *S. rosei*; S. exg., *S. exiguus*; S. fer., *S. fermentati*; As.=Antiserum absorbed in Exp. (No.); *=New antigen.

TABLE 2. RESULTS OF SLIDE AGGLUTINATION TESTS ON ANTISERA ABSORBED IN SEQUENCE WITH HEATED CELLS OF VARIOUS STRAINS AGAINST 15 DIFFERENT HEATED ANTIGENS (Experiment B).

of residual antibodies, homologous to antigen 10, remaining after the test performed in Experiment A. In experiment 9 (table 2) the absorbed antiserum was then reabsorbed with heated cells of *C. robusta,* which possesses antigen 10. The reabsorbed antiserum showed a positive reaction against only *T. glabrata.* This indicates the presence of a unique antigen in *T. glabrata,* and it was designated antigen 34, using Arabic numerals for its identification as was done for the thermostable antigens of the genus *Candida* previously reported. Similarly, in the other experiments shown in table 2, absorptions and slide agglutination tests were carried out successively to find a new antigen, but none was found. Many strains of different species besides the strains shown in tables 2-4, were used as antigens for slide agglutination tests. There were no discrepancies in the reaction results obtained by the slide agglutination method.

*T. stellata* var. *cambresieri* and *T. colliculosa* demonstrated the possession of thermostable antigen 24 as was found in *S. rosei. T. holmii* and *T. glabrata* showed the possession of antigen 6, which is present in *C. albicans.* Therefore, the former 2 species of *Torulopsis* can be placed in Group VII and the latter 2 species in Group I of the genus *Candida* (Tsuchiya *et al.*, 1959). Slide agglutination tests were carried out within each group or between serologically related species in order to find a new antigen, if present, and also to confirm their antigenic structures (table 3). No thermostable antigen, however, was found other than those antigens previously established. Furthermore, *T. stellata* var. *cambresieri* showed the absence of antigen 2 (table 3, expt. 20) since heated *C. albicans* antiserum absorbed with heated cells of *T. stellata* var. *cambresieri* showed positive agglutination reactions against every species having antigen 2. The residual antibodies homologous to antigen 2 could not be absorbed by the heated cells of *T. stellata* var. *cambresieri.* The discrepancy can be explained by assuming that the positive reactions shown in experiment A are due to the presence of residual antibodies homologous to other antigens. Because the absorbed antiserum homologous to antigen 2 was prepared from heated *C. albicans* antiserum absorbed with heated cells of *C. pseudotropicalis,* antibodies homologous to antigens 3, 4, 5, 6 and 7, besides antigen 2, would remain in the absorbed antiserum. Similar situations were observed in antiserum to antigen 2 of the other 3 species and also to antigen 4 of *T. glabrata.* These interpretations support the negative reactions of *S. bisporus*, which has no antigen 2.

Finally, antisera obtained from rabbits immunized with heated cells of 4 different species of *Torulopsis* were tested, without absorption, against heated cells of each species of the genus *Candida* by the slide agglutination method. Revised slide agglutination tests were also performed, and positive reactions were observed in both cases. Therefore, it can be concluded that the 4 species of the genus *Torulopsis* possess the common antigen 1.

*Thermolabile antigens.* Ten different monospecific antisera homologous to thermolabile antigens, *a* to *j*, were prepared as in the case of thermostable antigens, and slide agglutination tests were carried out with unheated cells against them. The 4 species of *Torulopsis*, however, lacked the thermolabile antigens which were described in the previous reports. Although in experiment

| *Exper. No.* | *Antiserum of heated cells* | *Heated cells for absorption* | *Heated cells used as antigens for agglutination* | | | | | | | | | | | | | | *Thermostable antigens homologous to antibodies remaining after absorption* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *T. st.* | *T. col.* | *T. hol.* | *T. glb.* | *C. alb.* | *C. pt.* | *C. kr.* | *C. pk.* | *C. glm.* | *C. pel.* | *S. bis.* | *S. ros.* | *S. exg.* | *S. fer.* | |
| 11—A | T. st. | T. col. | − | − | − | − | − | − | − | − | − | − | − | − | − | − | |
| —B | T. col. | T. st. | − | − | − | − | − | − | − | − | − | − | − | − | − | − | |
| 12—A | T. st. | S. ros. | − | − | − | − | − | − | − | − | − | − | − | − | − | − | |
| —B | S. ros. | T. st. | − | − | − | − | − | − | − | − | − | − | − | − | − | − | |
| 13—A | T. col. | S. fer. | − | − | − | − | − | − | − | − | − | − | − | − | − | − | |
| —B | S. fer. | T. col. | − | − | − | − | − | − | − | − | − | − | − | − | − | − | |
| 14—A | T. hol. | S. exg. | − | − | − | − | − | − | − | − | − | − | − | − | − | − | |
| —B | S. exg. | T. hol. | − | − | − | − | − | − | − | − | − | − | − | − | − | − | |
| 15—A | T. hol. | T. st. | − | − | + | + | + | + | − | − | − | − | + | − | + | − | (5), 6, 10, 26 |
| —B | T. st. | T. hol. | + | + | − | − | − | − | − | − | − | − | − | + | − | + | 24 |
| 16—A | T. hol. | T. col. | − | − | + | + | + | + | − | − | − | − | + | − | + | − | (5), 6, 10, 26 |
| —B | T. col. | T. hol. | + | + | − | − | − | − | − | − | − | − | − | + | − | + | 24 |
| 17—A | T. glb. | T. st. | − | − | + | + | + | ± | − | − | − | − | + | − | + | − | 6, 10, 34 |
| —B | T. st. | T. glb. | + | + | + | − | + | − | − | − | + | − | − | + | + | + | 4, 24 |
| 18—A | T. glb. | T. col. | − | − | + | + | + | + | − | − | − | − | + | − | + | − | 6, 10, 34 |
| —B | T. col. | T. glb. | + | + | + | − | + | − | − | − | + | − | − | + | + | + | 4, 24 |
| 19—A | T. glb. | T. hol. | − | − | − | + | − | − | − | − | − | − | − | − | − | − | 34 |
| —B | T. hol. | T. glb. | − | − | + | − | ± | − | − | − | ± | − | − | − | + | − | (4), (5), 26 |
| 20 | C. alb. | T. st. | − | − | + | + | + | − | + | + | + | + | − | − | + | − | 2, 5, 6, 7 |
| 21 | C. alb. | T. col. | − | − | + | + | + | − | + | + | + | + | − | − | + | − | 2, 5, 6, 7 |
| 22 | C. alb. | T. hol. | − | − | − | ± | + | − | − | + | + | − | − | − | − | − | 2, 7 |
| 23 | C. alb. | T. glb. | + | + | ± | − | + | − | − | ± | + | − | − | + | + | + | 2, 4, 5, 7 |

TABLE 3. RESULTS OF SLIDE AGGLUTINATION TESTS OF ABSORBED ANTISERA OBTAINED AFTER RECIPROCAL ABSORPTIONS WITHIN EACH GROUP OF ANTIGENICALLY RELATED SPECIES AGAINST 14 DIFFERENT HEATED ANTIGENS (Experiment C).

| *Exper. No.* | *Antiserum of formolized organisms* | *Heated cells for absorption* | *Thermolabile antigen* | *Unheated cells used as antigens for agglutination* | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *T. st.* | *T. col.* | *T. hol.* | *T. glb.* | *C. pt.* | *C. kr.* | *C. rob.* | *C. rug.* | *C. pul.* | *C. zey.* | *C. pk.* | *D. gl.* | *Sw. occ.* |
| 24 | T. st. | T. st. | | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 25 | T. col. | T. col. | | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 26 | T. hol. | T. hol. | | − | − | − | − | − | − | − | − | − | − | − | − | − |
| 27 | T. glb. | T. glb. | *k* | − | − | − | + | − | − | − | − | − | − | − | − | − |
| 28 | As(27) | T. glb.* | | − | − | − | − | − | − | − | − | − | − | − | − | − |

C. pul., *C. pulcherrima*; C. rug., *C. rugosa*; C. zey., *C. zeylanoides*; D. gl., *Debaryomyces globosus*; Sw. occ., *Schwanniomyces occidentalis*; * = Unheated cells for absorption.

TABLE 4.—RESULTS OF SLIDE AGGLUTINATION TESTS OF ANTISERA ABSORBED WITH HEATED AND UNHEATED CELLS OF THE SAME SPECIES AGAINST UNHEATED ANTIGENS (EXPERIMENT D).

D the 3 species of *Torulopsis* showed no thermolabile antigen, formolized *T. glabrata* antiserum, absorbed with heated cells of the same species, showed positive reaction against live cells of only *T. glabrata*. Consequently, it can be deduced that *T. glabrata* has an independent antigen, and the thermolabile antigen was designated as antigen *k*, following our system of labelling. The absorbed antiserum, which was reabsorbed with live cells of *T. glabrata*, showed negative reactions against live cells of every species tested, as was expected. Antigenic structures of the 4 species are shown in table 5.

| *Group* | *Species* | *Thermostable antigens* | | | | | | | | | | *Thermolabile antigens* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I | *C. albicans* | 1 | 2 | 3 | 4 | 5 | | | 6 | 7 | | |
| | *T. glabrata* | 1 | | 3 | | | 10 | | 6 | | 34 | *k* |
| | *T. holmii* | 1 | | 3 | (4) | (5) | 10 | | 6 | | 26 | |
| | *S. exiguus* | 1 | | 3 | 4 | (5) | 10 | | 6 | | 26 | |
| II | *C. pseudotropicalis* | 1 | | | | | (10) | | 8 | | | *a* |
| III | *C. krusei* | 1 | 2 | | | 5 | | | (11) | | | *b* |
| IV | *C. parakrusei* | 1 | 2 | 3 | | 5 | (14) | (15) | (13) | | | *c* |
| V | *C. guilliermondii* | 1 | 2 | 3 | 4 | | | | 9 | | | |
| VI | *C. pelliculosa* | 1 | 2 | | | | 14 | 15 | 16 | | 20 | |
| VII | *S. rosei* | 1 | | 3 | 4 | | | | 24 | | | |
| | *T. stellata* var. *cambresieri* | 1 | | 3 | 4 | | | | 24 | | | |
| | *S. fermentati* | 1 | | 3 | 4 | | | | 24 | | | |
| | *T. colliculosa* | 1 | | 3 | 4 | | | | 24 | | | |

TABLE 5. ANTIGENIC STRUCTURES OF VARIOUS SPECIES OF THE GENERA *Torulopsis*, *Candida* AND *Saccharomyces*.

An attempt was made to discover if the antigenic structures of other stock strains and isolates agreed with those of the standard. The antigenic structures of species cannot be used for classification unless the antigenic structures of every strain, other than the standards, correspond with those of the standard. Therefore, the biological characteristics of the test strains were examined, and then the existence of various antigens was tested by the slide agglutination method using monospecific antisera. All 5 strains of *T. holmii* tested demonstrated the possession of antigens 3, 4, 5, 6, 10 and 26, and all 33 strains of *T. glabrata* showed the existence of antigens 3, 6, 10 and 34. Two strains of *T. colliculosa* demonstrated the possession of antigens 3, 4 and 24; however, they might have been the same strains appearing in a different manner. Although the 2 strains of *T. colliculosa* were authentic, neither of them fermented nor assimilated maltose when tested by the auxanographic method. Nevertheless, assimilation of maltose was observed in both strains, when liquid media supplemented with vitamins was employed.

## DISCUSSION

In this study, *T. stellata* var. *cambresieri*, *T. colliculosa*, *T. holmii* and *T. glabrata* were examined for their specific antigens, and their antigenic structures

| *Source* | *Species* | *No. of strains* | *Biological characteristics* | | | | | | | | | | | *Positive slide agglutination against monospecific antiserum homologous to antigen* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Fermentation* | | | | | *Assimilation* | | | | | | |
| | | | *Glucose* | *Galactose* | *Sucrose* | *Maltose* | *Lactose* | *Glucose* | *Galactose* | *Sucrose* | *Maltose* | *Lactose* | *$KNO_3$* | |
| Stock | T. col. | 2 | + | − | + | − | − | + | − | + | + | − | − | 3, 4, 24 |
| Stock | T. hol. | 2 | + | + | + | − | − | + | + | + | − | − | − | 3, 4, 5, 6, 10, 26 |
| Mouth | T. hol. | 2 | | | " | | | | | " | | | | " |
| Faeces | T. hol. | 1 | | | " | | | | | " | | | | " |
| Stock | T. glb. | 3 | + | − | − | − | − | + | − | − | − | − | − | 3, 6, 10, 34, k |
| Mouth | T. glb. | 2 | | | " | | | | | " | | | | " |
| Faeces | T. glb. | 5 | | | " | | | | | " | | | | " |
| Vagina (1) | T. glb. | 13 | | | " | | | | | " | | | | " |
| Vagina (2) | T. glb. | 10 | | | " | | | | | " | | | | " |

+, acid and gas produced or assimilated. −, not fermented nor assimilated.

TABLE 6. BIOLOGICAL AND SEROLOGICAL CHARACTERISTICS OF STOCK STRAINS AND OF ISOLATES OF THE GENUS *Torulopsis* (EXPERIMENT E).

were established. Lodder & Kreger-van Rij (1952) state that these 3 species are the imperfect forms of *S. rosei*, *S. fermentati* or *S. exiguus*, respectively. The antigenic structures of these 3 *Saccharomyces* species were analyzed and reported by Tsuchiya, Fukazawa, Sato, Kawakita, Yonezawa & Yamase (1958). In comparing the results, the antigenic structure of *T. stellata* var. *cambresieri* was identical to that of *S. rosei*. This situation is also true for *T. colliculosa* and *S. fermentati*, and for *T. holmii* and *S. exiguus*. Therefore, it can be deduced that biological relationships, such as the perfect and imperfect forms, exist serologically among them. The 2 test strains of *T. colliculosa* did not show positive fermentative and assimilative reactions with maltose as demonstrated by *S. fermentati*. Therefore, these strains should at present be identified as *T. stellata* because of their similarity in biological characteristics. They show, however, positive assimilation reactions with maltose using liquid media supplemented with vitamins. Thus, it can be considered that serological characteristics are more stable than biological characteristics.

Seeliger (1958) examined serological relationships among many species of the genera *Torulopsis*, *Candida*, *Cryptococcus* and certain ascosporogenous yeasts. He indicated that most species of the genera *Torulopsis* and *Candida*, but not the genus *Cryptococcus*, have common antigens when examined by the agglutination and precipitation methods. Hasenclever & Mitchell (1960) studied the antigenic relationships of *T. glabrata* and 7 species of the genus *Candida*. However, they did not report on the antigenic structures of other species of *Torulopsis*. We therefore, investigated the antigenic structures of 4 species of *Torulopsis*. When the data of Hasenclever & Mitchell (1960) are compared with the results reported in this paper, a close agreement will be noted, if the designation of (−) or (+) is interpreted as either a negative or a positive reaction. We consider that minor discrepancies existing between the results are principally due to the difference in the methods employed for the preparation of antigens. For the immunization of animals, Hasenclever & Mitchell used antigens heated at 65°C. for about 6 hours, whereas we used antigens heated at 100° C. for 2½ hours. However, it is important to note that Hasenclever & Mitchell demonstrated the existence of a specific antigen in *T. glabrata* and this antigen corresponded to our thermostable antigen 34 and thermolabile antigen *k*. Therefore, *T. glabrata* can be identified rapidly and accurately with the aid of monospecific antiserum homologous to antigen 34, in addition to the antisera for antigens 6, 10 and *k*. The monospecific antiserum for antigen 34 can be preared from heated *T. glabrata* antiserum absorbed first with heated cells of *C. albicans* followed by reabsorption with heated cells of *C. robusta*.

Thirty-three strains of *T. glabrata* were examined serologically and biologically and an agreement was found between the results obtained. The same situation was observed in the cases of *T. holmii* and *T. colliculosa*, although the number of strains employed was few. Therefore, it can be concluded that the antigenic structures of species are not only adequate for the classification to genus but they are also useful for the basis of a rapid species identification. Antigenic analyses of the other species of the genus *Torulopsis* are in progress, and will be reported later.

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## ZUSAMMENFASSUNG

Antigenanalysen von 4 Arten der Gattung *Torulopsis* wurden mit Hilfe der Objektträger-Agglutinations-Methode durchegführt, und zwar unter Verwendung von monospezifischen und abgesättigter-Antiseren wie bei den Antigenen der Gattung *Candida*. Die Antigenformeln der einzelnen Arten wurden durch reziproke und aufeinander folgende Absorption und im Objektträger-Agglutinationstest gesichert. *Torulopsis stellata* var. *cambresieri* und *T. colliculosa* besitzen die thermostabilen Antigene 1, 3, 4 und 24, aber kein thermolabiles Antigen. Ihre Antigenformeln erwiesen sich als identisch mit deren von *Saccharomyces rosei*, bzw. *S. fermentati*, ungeachtet der biologischen Unterschiede zwischen beiden Arten. *Torulopsis holmii* hat die thermostabilen Antigene 1, 3, 4, 5, 6, 10 und 26, aber kein thermolabiles Antigen. Die Antigenformel dieser Art ist ähnlich der von *S. exiguus*. *Torulopsis glabrata* weist die thermostabilen Antigene 1, 3, 6, 10 und 34 und das thermolabile Antigen *k* auf. In einer vergleichenden Untersuchung stimmten die Antigenformeln einer Reihe von Stämmen mit denen der Standardstämme überein, wenn sie mit monospezifischen Antiseren analysiert wurden. Infolgedessen können die abgesättigter oder monospezifischen Antiseren für die rasche Artbestimmung verwendet werden und die Antigenformeln verschiedener Arten können als zuverlässige Kriterien bei der Klassifizierung dienlich sein.

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# SERIOLOGICAL STUDIES ON MYCOBACTERIA, *NOCARDIA, ACTINOMYCES,* AND FUNGI*

M. L. Bevis

*Department of Medical Microbiology, Univ. of Wisconsin, Madison, Wisconsin*†

Immunization of rabbits with concentrated antigens combined with an adjuvant was effective in producing precipitating antibodies against B.C.G., *Nocardia, Actinomyces,* and several dermatophytes. Antigenic analysis indicated that the organisms fall into 3 groups comprising B.C.G., *Nocardia,* and *Streptomyces* in one, *Actinomyces* in another, and the dermatophytes in the third. Fuller extracted antigens were more useful in detecting precipitins than were antigens extracted with a ball machine.

Hyperimmune sera has been successfully used in the identification and classification of bacteria showing morphological and cultural similarities. On the other hand, agglutination (Fritze, 1908; Colebrook, 1920; Erickson, 1940; Ludwig & Hutchison, 1949; Slack, Ludwig, Bird & Canby, 1951; Seeliger, 1954) and complement-fixation tests (Claypool, 1913; Nelson & Henrici, 1922; Goyal, 1938; Seeliger, 1954) with the fungi and actinomycetes have yielded conflicting data due to their weak antigenicity and the difficulty in preparing suitable antigens for the tests. Precipitin tests have been used with actinomycetes (Ludwig & Hutchison, 1949) and dermatophytes (Sharp, 1941) and in both groups considerable cross-reactions occurred. In an effort to circumvent these problems concentrated antigens were injected in combination with an adjuvant and the precipitin ring test was used to detect the presence of antibody.

## Materials and Methods

### *Cultures*

*Actinomyces bovis* (ATCC) #8374, strain 38411, human, non-acid-fast.

*Actinomyces bovis* (ATCC) #8374, bovine, non-acid-fast.

*Actinomyces bovis,* Dr. Johnson collection, Dermatology Dept., Wisconsin General Hospital.

*Mycobacterium tuberculosis* (B.C.G. strain), Tice Laboratories, Chicago, Illinois.

*Nocardia asteroides* (ATCC) #8408, aerobic, non-acid-fast.

*Nocardia asteroides* (ATCC) #9970, aerobic, non-acid-fast.

*Nocardia minima* (ATCC) #8674, aerobic, few acid-fast granules.

*Streptomyces gypsoides,* non-acid-fast, Dr. MacCoy, Bacteriology Dept., University of Wisconsin.

Strains of *Trichophyton rubrum, Epidermophyton floccosum* and *Microsporum audouini* were obtained from the collection of Dr. Johnson, Department of Dermatology, Wisconsin General Hospital.

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† Present address, Dept. of Microbiology, Seton Hall, College of Medicine and Dentistry, Jersey City, New Jersey.

### *Antigens*

In preparing antigens for immunization the cultures were grown as indicated below, centrifuged, the sediment washed 3 times in normal saline, and killed by evaporation to dryness at 37° C. after the addition of 10 volumes of acetone. The dried organisms were checked for viability, and stored at 4°C. until needed.

GROWTH MEDIA*

| *Organism* | *Stock cultures* | *Large quantities* | *Conditions of incubation* |
|---|---|---|---|
| *Actinomyces* | Alternate transfers on 1% dextrose broth and agar | 1% dextrose broth with 0·1% Na thioglycollate† | 2 weeks at 37°C. anaerobically |
| *Nocardia* | Alternate transfers on 1% dextrose broth and agar | 1% dextrose broth† | 2 weeks at 37°C. aerobically |
| *Streptomyces* | Alternate transfers on 1% dextrose broth and agar | 1% dextrose broth† | 2 weeks at 37°C. aerobically |
| *Mycobacterium* | Petragnani slants | Petragnani slants | 6 weeks at 37°C. |
| *Trichophyton* | 1% dextrose agar | 1% dextrose broth | 2-3 weeks at 23°C. |
| *Epidermophyton* | 1% dextrose agar | 1% dextrose broth | 2-3 weeks at 23°C. |
| *Microsporum* | 1% dextrose agar | 1% dextrose broth | 2-3 weeks at 23°C. |

* In addition, all of the organisms were grown in Liquid T.B.C. medium (Albimi Laboratories, Brooklyn, N.Y.), a modification of Dubos (1947) liquid medium. In the case of *Actinomyces* 0·1% Na thioglycollate was added.

† Neutralized frequently during growth with sterile 1N NaOH to roughly pH 6·7, phenol red indicator.

In the precipitin ring tests several different antigens were employed.

*Ball-machine extracted antigen*: Dried organisms (20-30 mg.) were placed in 2·5 × 16 cm. steel tubes containing small steel balls. These were immersed in a dry ice—95% alcohol bath for 10 minutes and then immediately placed on the shaking machine which was set to deliver approximately 500 vertical strokes per minute (6 cm. length). Shaking was allowed to proceed until friction brought the tubes to room temperature. The tubes were removed, chilled again, and the process repeated until 20 minutes of actual shaking time elapsed. The tubes were washed with 3 ml. of saline, the suspension centrifuged, and the clear supernate used as the antigen.

Assay of B.C.G., *A. bovis, T. rubrum,* and *E. floccosum* antigens for nitrogen content by the micro-Kjeldahl method gave values of 0·6-1·1, 0·4-0·6, 0·35, and 0·2 mg./ml. respectively.

*Fuller extract antigen*: Dried organisms (40 mg.) were extracted with formamide according to the technique of Fuller (1938). Efforts to get the final acetone precipitate to dissolve in neutralized saline were facilitated by holding the material overnight at 4° C. with occasional shaking. After centrifugation the clear supernate was used as the antigen.

*Lancefield extract antigen*: With *A. bovis* only, 80 mg. of the dried organisms were extracted according to the method of Lancefield (1933).

*Culture filtrate antigen*: Flasks of 4% dextrose broth containing 1% neopeptone were heavily inoculated with the fungus, incubated for 3 weeks at room temperature, the culture centrifuged, and the supernate used as antigen.

### *Antisera*

After grinding 60-95 mg. of the dried organisms in a mortar with sand, the powder was suspended in 20 ml. of adjuvant containing equal parts of lanolin and mineral oil, (Ramon, Lemetayer & Richow, 1935) held in a water bath at 40-45°C. to reduce the viscosity, and injected subcutaneously in 4 or 5 sites in rabbits anaesthetized with Nembutal. The serum of each rabbit was obtained before immunization and at frequent intervals after immunization. When there appeared to be no further increase in precipitin content, the animals were fasted for 24 hours, exsanguinated and the serum separated and stored at −27° C.

### *Precipitin Tests*

Antiserum was added to a depth of 1 cm. in capillary tubes 3 mm. diameter and 2 cm. long which were set up in plasticine and the antigen overlaid carefully to form a

| *Organism* | *Designation of rabbit* | *Weight of organisms injected in mg.* | *Days serum obtained* | *Precipitin ring titer* |
|---|---|---|---|---|
| | | | | Ball machine antigen |
| *A. bovis* | 1 | 45 | 28 | 1:8 |
| | | 30† | 34 | 1:100 |
| | | | 57 | 1:100 |
| | 2 | 35 | 28 | 1:2 |
| | | 100† | | Died |
| | 3 | 12 | 28 | 0 |
| | | 30† | 34 | 1:100 |
| | | | 57 | 1:100 |
| B.C.G. | 1 | 65 | 20 | 1:1000 |
| | 2 | 65 | 20 | 1:1000 |
| | 3 | 65 | 20 | 1:1000 |
| *N. asteroides* | 1 | 50 | 30 | 1:10 |
| | 2 | 30 | 30 | 1:100 |
| | 3 | 75 | 43 | 1:100 |
| | | | | Fuller extract antigen |
| *T. rubrum* | 1 | 80 | 67 | 0 |
| | | 50 | 100 | 0 |
| | | 20‡ | 181 | 1:1 |
| | 2 | 80 | 67 | 0 |
| | | | 100 | 1:4 |
| *M. audouini* | 1 | 60 | 67 | 0 |
| | | | 100 | 1:2 |
| | 2 | 95 | 52 | 1:1 |
| *E. floccosum* | 2 | 80 | 56 | 1:1 |

Except where noted the antigens were injected as indicated in Materials and Methods.

† Injected intravenously.

‡ Wet weight in viable organisms injected intraperitoneally.

TABLE 1.—PRECIPITIN RING TITERS

sharp interface. The time required for a turbid ring to form at the interface was noted. The test was recorded as negative if a ring did not form in 2 hours at room temperature. Saline and media controls were included with each antiserum.

## RESULTS

The data included in table 1 indicate that relatively high titers of demonstrable precipitins were formed in rabbits injected with *A. bovis*, B.C.G., and *N. asteroides* antigens combined with an adjuvant. Similar preparations of *T. rubrum*, *M. audouini*, and *E. floccosum* also produced detectable precipitates but at much lower titer. Subsequent experiments were concerned with examining the sera for cross-reactions before and after absorption with homologous and heterologous antigens.

The results of precipitin and cross-precipitin tests are shown in table 2. It is apparent that the organisms fall into 3 groups, one comprising B.C.G., *Nocardia*, and *Streptomyces*, another the *Actinomyces* and the third the dermatophytes. The speed with which the antigen and antibody molecules combine to form a turbid ring in the various tests is not surprising in view of the antibody titers included in table 1. In most cases the reactions appeared earlier with the Fuller extracted

| | *ANTIGENS*† | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ANTISERA | *B.C.G.* (*B*) | *B.C.G.* (*F*) | *N. asteroides #9504* (*B*) | *N. asteroides #9504* (*F*) | *N. asteroides #9970* (*B*) | *N. asteroides #9970* (*F*) | *N. minima* (*B*) | *N. minima* (*F*) | *S. griseus* (*B*) | *S. griseus* (*F*) | *A. bovis #8373* (*B*) | *A. bovis #8373* (*F*) | *A. bovis #8374* (*B*) | *A. bovis #8374* (*F*) | *A. bovis (Johnson's)* (*B*) | *A. bovis (Johnson's)* (*F*) | *T. rubrum* (*B*) | *T. rubrum* (*F*) | *M. audouini* (*B*) | *M. audouini* (*F*) | *E. floccosum* (*B*) | *E. floccosum* (*F*) |
| B.C.G. Pooled | 5 | 5 | 20 | 5 | 20 | 5 | 30 | 5 | 10 | 5 | — | — | — | — | — | — | — | — | — | — | — | — |
| *N. asteroides #9504* Pooled | 5 | 5 | 20 | 5 | 20 | 5 | 20 | 5 | 5 | 5 | — | — | — | — | — | — | — | — | — | — | — | — |
| *A. bovis #8374* Pooled | — | — | — | — | — | — | — | — | — | — | 10 | 10 | 10 | 10 | 20 | 20 | — | — | — | — | — | — |
| *T. rubrum* 1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 120 | 90 | — | 90 | — | 30 |
| *T. rubrum* 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 120 | 90 | 120 | 90 | 120 | 30 |
| *M. audouini* 1 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 120 | 60 | 120 | 60 | — | 30 |
| *M. audouini* 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 90 | 30 | 120 | 30 | — | 30 |
| *E. floccosum* 2 | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 90 | — | 120 | 120 | 30 |

(*B*) Ball-Machine extract.
(*F*) Fuller extract

The figures in the table refer to the time in minutes required for the formation of a turbid ring at the interface between undiluted antibody and undiluted antigen. A negative sign indicates that no reaction was observed in 120 minutes.

† All of the antigens were prepared from organisms grown in liquid TBC medium.

TABLE 2.—PRECIPITIN RING TESTS

than with the ball-machine extracted antigens, and the latter occasionally failed to detect precipitins in the dermatophyte group. Although the data recorded in table 2 involved the use of antigens prepared from organisms grown in liquid TBC medium, similar results were obtained with those organisms which could be cultured in 1 % dextrose broth. On the other hand, unusually strong cross-reactions with the dermatophytes developed when culture filtrate antigens of organisms grown in neopeptone dextrose broth were examined. Since neopeptone is rich in blood group "A" substance, one of the antisera was absorbed with a crude preparation of "A" substance made according to the procedure of Gerheim, Berkut & Gerheim (1949). When the absorbed serum was tested, a positive ring test developed with a Fuller-extracted antigen but not with the culture filtrate antigen. Finally, it was noted that all the dermatophyte antisera as well as several normal rabbit sera produced strong reactions with "A" substance or group "A" cells. Failure to observe reactions with uninoculated neopeptone dextrose broth suggested that group "A" substance was liberated into the medium during the growth of the organisms.

| *Antisera* | *Absorbed with* † | *Fuller-Extract Antigen* | | | | |
|---|---|---|---|---|---|---|
| | | *B.C.G.* | *Nocardia asteroides #9504* | *Nocardia asteroides #9970* | *Nocardia minima* | *Streptomyces gypsoides* |
| B.C.G. | — | 5 | 5 | 5 | 5 | 5 |
| | B.C.G. | 5 | – | – | – | – |
| | *N. asteroides* #9504 | 5 | – | – | – | 5 |
| *N. asteroides* #9504 | — | 5 | 5 | 5 | 5 | 5 |
| | *N. asteroides* #9504 | – | 5 | 5 | – | – |
| | B.C.G. | – | – | – | – | – |

The figures in the table refer to the time in minutes required for the formation of a turbid ring at the interface between undiluted antibody and undiluted antigen. A negative sign indicates that no reaction was observed in 120 minutes.

† Killed, dried, organisms prepared as described in Materials and Methods Section.

Table 3.—Precipitin Reactions with Absorbed and Unabsorbed B.C.G. and *Nocardia* Antisera

The antigenic relationships between *B.C.G.*, *Nocardia,* and *Streptomyces* are more adequately revealed by the data included in table 3. A single absorption of B.C.G. antisera with B.C.G. antigen completely removed cross-reactions with the *Nocardia* and *Streptomyces* antigens, while not detectably altering the homologous reaction. Absorption of B.C.G. antisera with *N. asteroides* #9504, while abolishing the cross-reactions with the *Nocardia* antigens, did not measurably alter the reaction with *Streptomyces* and B.C.G. antigens. Similar absorption of *N. asteroides* #9504 antisera with the homologous antigen removed cross-reactions with B.C.G., *Streptomyces,* and *N. minima* antigens, but did not detectably impair the reactions with *N. asteroides* #9504 and #9970. Absorption of the *Nocardia* antisera with B.C.G. completely removed the homologous as well as the heterologous reactions.

Among the dermatophytes tested antigenic relationships are extremely close judging by the methods employed (table 4). Cross-absorption of antisera with

| *Antisera* | *Absorbed with*† | *Fuller-Extract Antigens* | | |
|---|---|---|---|---|
| | | *T. rubrum* | *M. audouini* | *E. floccosum* |
| *T. rubrum* | — | 90 | 90 | 30 |
| *T. rubrum* | *T. rubrum* | — | — | — |
| | *M. audouini* | — | — | — |
| | *E. floccosum* | — | — | — |
| *M. audouini* | — | 30 | 30 | 30 |
| *M. audouini* | *M. audouini* | — | — | — |
| | *T. rubrum* | — | — | — |
| | *E. floccosum* | — | — | — |
| *E. floccosum* | — | 90 | 120 | 30 |
| *E. floccosum* | *E. floccosum* | — | — | — |
| | *M. audouini* | 120 | — | 120 |
| | *T. rubrum* | — | — | — |

The figures in the table refer to the time in minutes required for the formation of a turbid ring at the interface between undiluted antibody and undiluted antigen. A negative sign indicates that no reaction was observed in 120 minutes.

† Killed, dried, organisms prepared as described in Materials and Methods Section.

TABLE 4.—PRECIPITIN REACTIONS WITH ABSORBED AND UNABSORBED SERA AGAINST DERMATOPHYTES

heterologous antigens removed the reacting antibodies with the exception of *E. floccosum* antiserum absorbed with *M. audouini* antigen. Since the reactions occurred with *T. rubrum* and *E. floccosum* but not with *M. audouini* antigen, they are a reflection of incomplete absorption of the serum by *M. audouini* antigen.

## DISCUSSION

The findings of this investigation appear to confirm the precipitin and agglutination reactions of Ludwig & Hutchison (1949), complement-fixation and agglutination reactions of Seeliger (1954) and fluorescent antibody reactions of Slack, *et al* (1961), all of whom found only slight or no cross-reactions between various species of *Nocardia* or *Streptomyces* and *Actinomyces*.

These results also agree with the complement-fixation and agglutination observations of Seeliger (1954) showing cross-reactions between Mycobacteria and *Nocardia*.

If it is assumed that the acid-fast *Actinomyces* referred to in the work of Nelson & Henrici (1922) are actually organisms currently classified as *Nocardia*, then the precipitation studies described here would agree with their results. By complement-fixation tests, they demonstrated a lack of cross-reaction among a number of *Actinomyces* and *Nocardia*(?) or *M. tuberculosis* but found cross-reaction between *M. tuberculosis* and certain *Nocardia* (?).

There is conflict, however, between the present observations and agglutination reactions of Fritze (1908), and complement-fixation reactions of Goyal (1938), both of whom found cross-reactions between *Actinomyces* and Mycobacteria. Slack *et al.* (1951) has also shown low-titer cross-reactions between *Nocardia* and *Actinomyces* which were not confirmed by the precipitation methods reported herein.

It should be noted that, in this discussion, the various organism of the order Actinomycetales have been referred to by the nomenclature used by the authors. It is possible that some of the conflicting results may be due to problems in taxonomy and nomenclature which still await clarification.

Results among the dermatophytes agree with those of Sharp (1941) whose precipitin tests showed cross-reactions among certain strains of *Trichophyton* and *Microsporum.*

## Résumé

L'immunisation de lapins à l'aide d'antigènes concentrés accompagnés d'un adjuvant est efficace et permet d'obtenir des anticorps précipitants contre le B.C.G., des *Nocardia, Actinomyces* et plusieurs dermatophytes.

L'analyse antigènique montre que les organismes étudiés se classent en trois groupes: B.C.G., *Nocardia* et *Streptomyces* dans l'un, *Actinomyces* dans un second et les dermatophytes dans un troisième. Les antigènes extraits à la technique de Fuller sont préférables aux antigènes extraits par moulin à billes pour détecter les précipitines.

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# A PROPOS DES MYCETOMES À *NOCARDIA*

P. Destombes, F. Mariat, Olga Nazimoff et Jacqueline Satre

*Services d'Anatomie pathologique et de Mycologie, Institut Pasteur, Paris.*

Les auteurs ont figuré les morphologies des "petits grains" de mycétomes qu'ils ont reçus d'Afrique, du Vietnam et du Pacifique au cours des dernières années. Ces agents, rares dans la zone sahélienne des mycétomes, deviennent prédominants dans la zone équatoriale africaine. Les AA. ont tenté de préciser la part qui revient dans ce syndrome à *Nocardia asteroides* ou à *N. brasiliensis*. Ils décrivent les aspects morphologiques obtenus expérimentalement avec des souches connues de ces 2 espèces.

Ces recherches sont en faveur du rôle prédominant de *N. brasiliensis* dans l'étiologie des mycétomes à petits grains. Mais *N. asteroides* est aussi très probablement en cause dans un certain nombre de cas. La question requiert encore de nouvelles recherches, étant donnés les doutes qui subsistent encore et l'importance grandissante de cette catégorie de mycétomes.

Un certain nombre de germes pathogènes ont la propriété curieuse de s'organiser dans les tissus en masses compactes dénommées "grains". Ceux-ci représentent un mode très particulier de développement *in situ*. Ils offrent une extraordinaire résistance à la réaction tissulaire, pourtant puissante, qui s'exerce sur eux, d'où le caractère chronique de la maladie qu'ils traduisent. L'extension de l'infection dans les tissus s'effectue par fragmentation des grains et essaimage de proche en proche. La propagation à distance par voie lymphatique est parfois constatée en clinique.

Ce mode granulaire de croissance s'observe exceptionnellement chez le Staphylocoque et à titre rare chez les mycobactéries (Levaditi, Bretey & Pick, 1957) il devient constant dans le groupe des actinobacilles; il est fréquent chez les actinomycètes et dans certains groupes de champignons qui sont les agents des mycétomes.

Les actinomycètes pathogènes comportent le groupe anaérobie de l'*Actinobacterium israelii* agent de l' "actinomycose" classique.

A côté de ce genre anaérobie, d'autres, aérobies, se répartissent en 2 groupes :

(1) les *Streptomyces*, avec les 3 espèces pathogènes jusqu'ici connues: *S. madurae*, *S. pelletieri* et *S. somaliensis* (*).

Les caractères des grains de ces 3 *Streptomyces* sont maintenant précisés et parfaitement spécifiques.

(2) les *Nocardia* avec les 2 espèces pathogènes, *N. asteroides* et *N. brasiliensis*.

*N. brasiliensis* est l'agent prédominant des mycétomes au Mexique et en Amérique du Sud. Il gagne parfois le relais ganglionnaire lymphatique, mais n'est pas connu avec certitude comme agent de nocardioses viscérales et généralisées. Dans les tissus il entraîne la formation de petits grains(†).

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(*) D'après quelques cas de nos archives histologiques, d'autres agents non encore identifiés (*Nocardia?*) sont en cause en quelques points d'Afrique et de Madagascar.

(†) Sous le vocable de "mycétomes à petits grains", que nous utilisons dans ce mémoire, nous entendons des mycétomes dont les formations parasitaires agrégées n'excèdent pas 200 μ. Par analogie de formes et de structures, nous employons les mêmes termes pour les grains expérimentaux que nous avons obtenus bien qu'ils atteignent fréquemment des tailles plus élevées. Des travaux effectués à ce jour montrent que les mycétomes à petits grains doivent être attribués au genre *Nocardia*.

*N. asteroides,* agent habituel des nocardioses viscérales existe dans les réactions granulomateuses sous forme de filaments isolés. Cet actinomycète peut être à l'origine de mycétomes et se présenter alors dans les lésions sous forme de petits grains. Certains auteurs n'admettent pas qu'il puisse en être ainsi. Par inoculation à l'animal les 2 espèces forment des grains parfois très nombreux.

Le diagnostic étiologique d'un mycétome repose sur la détermination du germe obtenu en culture mais aussi sur l'aspect histologique de ses grains. Un certain nombre de mycétomes à *N. brasiliensis* et *N. asteroides* ont été déterminés selon ces 2 méthodes, tels sont :

—ceux du Mexique où *N. brasiliensis* est largement prédominant (Latapi, 1959).

—ceux du Congo ex-Belge où Vanbreuseghem, Courtois, Thys & Doupagne (1956) ont par 2 fois obtenu la même espèce,

—enfin ceux d'Amérique du Nord où, à côté de *N. brasiliensis,* Conant (1955) a décrit un mycétome à *N. asteroides.*

Malheureusement l'utilisation conjointe des 2 méthodes n'est pas toujours possible et on ne dispose souvent que de documents histologiques. C'est le cas pour la totalité de notre matériel. Le problème serait simple si les aspects histologiques étaient constants pour une espèce donnée comme il en est des *Streptomyces* pathogènes. Mais il existe pour les grains de *N. brasiliensis* et *N. asteroides* des variations qui ne permettent pas un diagnostic histologique facile de l'espèce en cause.

Le but principal de ce travail est de tenter d'identifier les mycétomes que nous avons eu à examiner en comparant la morphologie de leurs grains' d'une part à celles des mycétomes sûrement diagnostiqués, d'autre part à celles des grains expérimentaux obtenus chez l'animal par l'inoculation de souches connues.

## Observations

### I—*Aspect Histologique des Grains de Mycétomes à Etiologie Connue*

### (a) *Mycétomes à N. brasiliensis*

La planche I reproduit les aspects les plus nets des grains de mycétomes déterminés avec certitude au Mexique et au Congo ex-belge. Nous remercions vivement les Docteurs P. Lavalle, R. Vanbreuseghem et G. Courtois de nous avoir adressé leurs documents.

(1) *Mycétomes mexicains* (Y 1186 et BB 471)

Au faible grossissement, les grains de Y 1186 apparaissent au sein des réactions purulentes comme des masses arrondies, surtout ovalaires, de petite taille, de 40-70 μ de petit diamètre et de 70-100 μ de grand diamètre. Ils sont cernés par une réaction massuée éosinophile développée et remarquablement dessinée. Si la coupe ne passe pas par le centre du grain, celui-ci peut sembler n'être constitué que par ces massues coupées perpendiculairement ou radiairement selon les points. Les massues ne sont pas fuchsinophiles et apparaissent donc bleues par la méthode de Ziehl; elles prennent une teinte brun-violacé au Ziehl-Gram. Des plages homogènes, mauve-lilas à l'hémalun-éosine-safran sont souvent incluses dans la substance éosinophile du matériel massué. Moins de la moitié des grains montre la présence de germes. On voit alors, disséminées irrégulièrement, des plages de granulations hématéiphiles, fines, cocciformes, qui ne prennent qu'exceptionnellement l'aspect filamenteux. Au Ziehl-Gram, au contraire, on observe des filaments fortement Gram-positif, typiquement actinomycosiques; ils sont de dimensions variables, souvent allongés, avec des ramifications à angle droit et des renflements intercalaires, latéraux et terminaux. La mise en évidence de leur semi-acido-résistance est très délicate sur coupe et demande une différenciation acide ménagée.

Dans le second cas mexicain (BB 471 = 1231 du C.D.P.* Mexico), les grains sont plus polymorphes, certains sont superposables aux précédents (Pl. I, fig. 1) mais il en existe de très allongés, d'aspect vermiforme plus ou moins coudés (Pl. I, fig. 2). En longueur, ces grains peuvent atteindre 300-400 μ, leur largeur restant réduite à 15-20 μ. Cette forme se retrouve sur des coupes successives, il s'agit donc de lames bactériennes à courbures irrégulières et à bords souvent repliés. Les massues, dans les grains vermiformes, sont rares et localisées au niveau des courbures ou des extrêmités.

(2) *Mycétomes congolais* (Ngengelé coupes Z 107 et Z 108 = A 10299 Dr. Courtois)

Les grains observés dans ces 2 biopsies d'un même cas sont nombreux. Leur taille est très variable: si certains grains n'ont que 50-70 μ, d'autres atteignent 100-175 μ.

A côté de grains trapus et massifs, il existe des arceaux et des festons bourgeonnants qui s'isolent du grain dont ils sont nés. Il en résulte des grains multiples au sein d'un même abcès. On n'observe pas d'aspect vermiforme (Pl. I, fig. 3-6).

Leur structure comporte 3 couches concentriques de l'extérieur vers l'intérieur :

(a) une couronne massuée périphérique où les massues sont bien formées, piriformes, mais toujours plus courtes que dans les formes mexicaines.
(b) un feutrage périphérique hématéiphile de germes qui à l'hémalun sont surtout granulaires et rarement filamentés. Au contraire, les germes prennent le Gram et leur forme est typiquement actinomycosique. Ce feutrage est parfois dédoublé par un espace clair.
(c) le centre du grain est occupé par des cellules inflammatoirs (polynucléaires altérés) qui sont en continuité par un hile avec l'infiltrat purulent environnant.

### (b) *Mycétomes à N. asteroides* (*Conant*)

Dans les coupes que nous devons à l'amabilité du Dr. N. F. Conant (Y 829) les grains de ce mycétome se présentent en masses ovoïdes ou étirées, parfois à extrémités rebroussées. Si leur longueur varie de 60-120 μ, leur largeur moyenne est de 20 μ et ne dépasse pas 30 μ. Une mince couronne éosinophile, à courtes massues, les enveloppe presque totalement. Ils sont formés d'un semis de granulations légèrement hématéïphiles mais dans l'ensemble peu nettes. Au Ziehl, on ne décèle pas d'acido-résistance, les filaments et granulations apparaissent en bleu. La couronne est faiblement fuchsinophile, mais les petites massues sont bleues.

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* Centro Dermatologico Pascua, Mexico 7, D.F.

## II—"*Petits Grains*" *Expérimentaux*

Hormis une série d'expériences où le lapin (inoculé par voie intraveineuse) a été utilisé, ce sont toujours les cobayes ou les souris (inoculés par voie intrapéritonéale) que nous avons employés. Dans tous les cas l'actinomycète à injecter est prélevé dans des tubes de culture, sur milieu solide et il est broyé aussi finement que possible au mortier. Dans certaines expériences, nous avons associé à l'actinomycète soit de l'huile de paraffine, soit du bacille tuberculeux tué. Ces adjuvants n'ont pas permis d'obtenir de différences notables comparativement aux témoins. Il nous a paru, en revanche, indispensable d'inoculer une suspension très riche d'actinomycète (5-10 mg. de germes frais) si l'on désire obtenir la formation de grains (Murray, Spooner & Walker, 1960).

Des souches des 2 espèces pathogènes de *Nocardia* (*N. asteroides* et *N. brasiliensis*) ont été utilisées.

*N. asteroides*

Les 2 souches (321 et 318) inoculées proviennent toutes deux de nocardioses

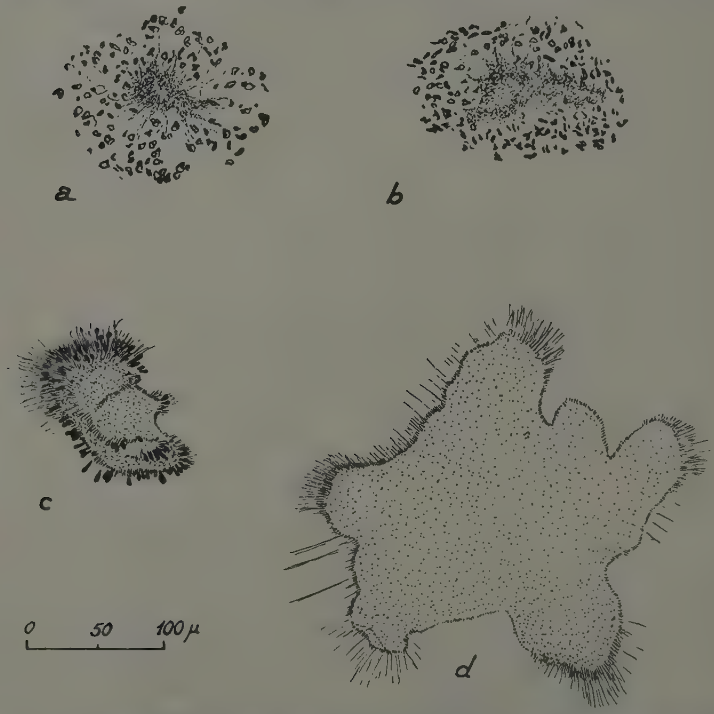

Figure 1.—*a*, *b* : morphologie de grains obtenus à la suite d'inoculation au cobaye de la souche 321 de *N. asteroides* en suspension dans de l'huile de paraffine.

*c* : grain massué observé chez le cobaye inoculé avec *N. asteroides* souche 318. Il est comparable à certains grains de mycétomes spontanés à *N. brasiliensis*.

*d* : autre morphologie de grain obtenu dans les mêmes conditions.

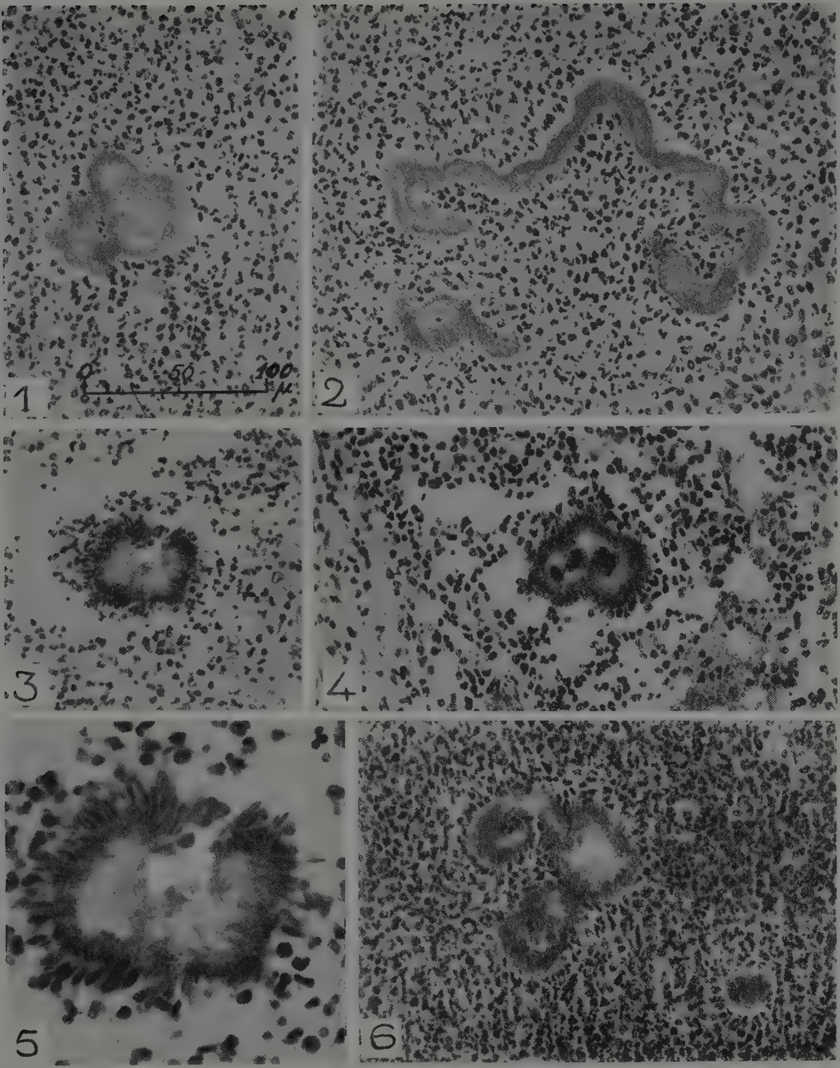

PLANCHE I : Mycétomes d'étiologie connue

Figure 1.—Mycétome à *N. brasiliensis* (P. Lavalle, Mexico) BB 471 Hémalun éosine safran. 260 ×.

Figure 2.—même cas, grain vermiforme. 260 ×.

Figure 3.—Mycétome à *N. brasiliensis* (cas 1 de Vanbreuseghem *et al.*) Grain massué. Ziehl Gram. 260 ×.

Figure 4.—Même malade que fig. 3 (2 ème biopsie). Grain légèrement bourgeonnant. Hémalun. 260 ×.

Figure 5.—id. fig. 3, Filaments et granulations denses dans le feutrage périphérique, lâches au centre. 610 ×.

Figure 6.—Même malade; autre aspect morphologique. Hémalun. 260 ×.

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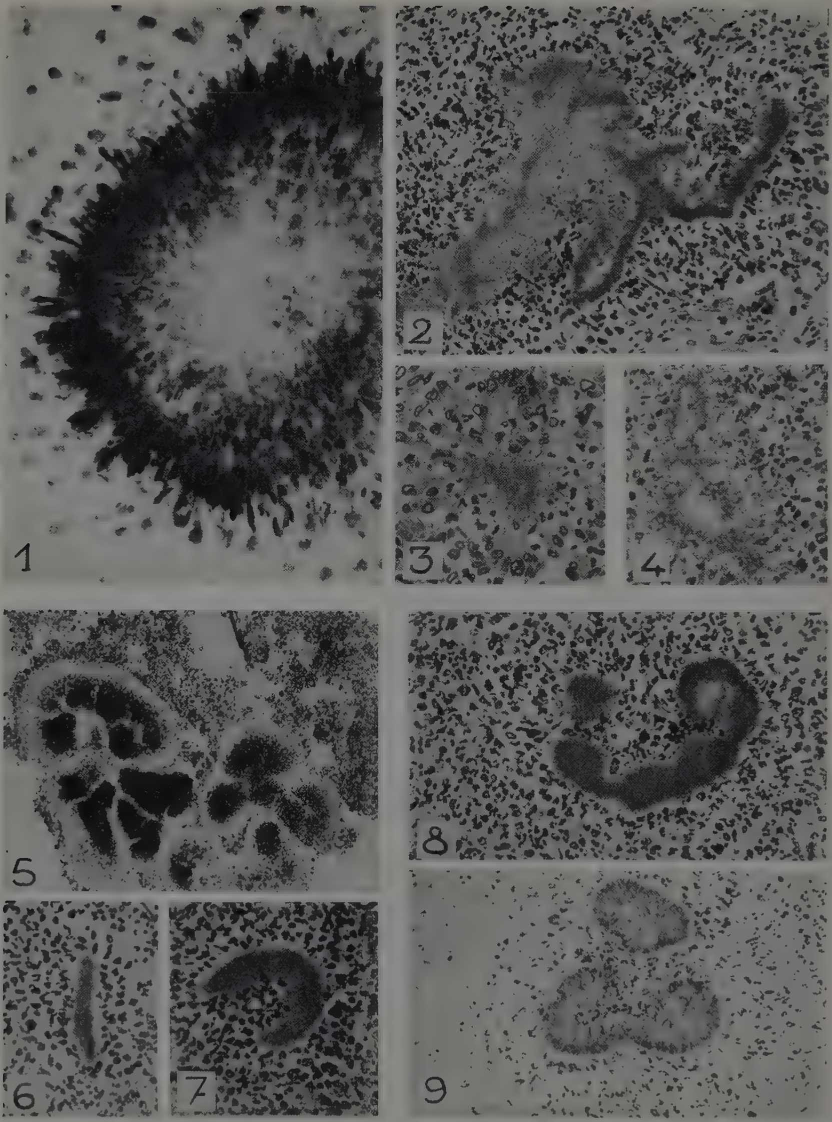

PLANCHE II : Grains expérimentaux

Figure 1.—Grain massué dans le péritoine d'un cobaye inoculé avec *N. asteroides* 318. Ziehl-bleu. 610 ×.

Figure 2.—Grain irrégulier, rubanné dans le péritoine d'une souris inoculée avec *N. asteroides* 318 en association avec du bacille tuberculeux tué. Hémalun-éosine-safran. 260 ×.

Figures 3 et 4—Grains dans le péritoine d'un cobaye inoculé avec *N. asteroides* souche 321 en suspension dans de l'huile de paraffine. Hémalun-éosine-safran. 260 ×.

Figure 5. —Grain à l'examen direct dans une goutte de potasse (*N. brasiliensis* 531 inoculé à la souris en association avec du bacille tuberculeux tué). 135 ×.

Figures 6, 7, 8 et 9—Grains de divers aspects dans le péritoine de souris inoculées avec *N. brasiliensis* 531 avec du bacille tuberculeux tué. Fig. 6, 7 et 8 : Hémalun—fig. 9 : Ziehl-bleu. 260 ×.

*To face page* 165)

viscérales où les filaments étaient toujours dispersés et jamais agrégés. Il est intéressant de constater qu'expérimentalement ces 2 souches ont entrainé chacune la formation de grains. Mais ils sont constamment accompagnés de filaments isolés dans la réaction purulente, fait que nous ne constatons pas avec *N. brasiliensis*. Ces 2 souches se sont avérées fortement pathogènes, C'est ainsi qu'avec la souche 318, 6 sur 12 des animaux inoculés sont morts avant le terme fixé pour l'expérience.

Les grains obtenus sont plus petits avec la souche 321 qu'avec la souche 318. Ce sont, avec la première, soit des petites touffes très astéroïdes de filaments radiés (30-60 μ) (fig. 1 *a*, *b*; Pl. II fig. 3, 4), soit des grains arrondis ou anguleux de 10-100 μ.

Avec la seconde (318) les masses sont plus volumineuses, régulières ou irrégulières (fig. 1 *d*; Pl. II, fig. 2) atteignant, selon l'animal, 60-150 μ chez la souris ou 100-300 μ chez le cobaye.

Certains des grains obtenus avec la souche 318 sont pourvus de massues (Fig. 1 *c*; Pl. II, fig. 1); celles-ci sont éosinophiles, et parfois acido-résistantes et leur taille peut atteindre 10 μ de longueur sur 3 μ de largeur. Elles ont parfois un filament comme axe.

Les filaments se présentent à la coloration de routine sous forme de ponctuations, mais la technique de Gram montre bien leur morphologie actinomycosique. Ils sont partiellement acido-résistants avec la souche 321, mais ne le sont pas avec la souche 318. Celle-ci, inoculée au lapin par voie intraveineuse, provoque la formation de grains souvent massués, essentiellement dans le poumon. Mais ces grains, en 3 mois, se résorbent progressivement, puis disparaissent totalement. Les massues qui ne sont pas acido-résistantes au 4 ème jour le deviennent au 16 ème jour.

*N. brasiliensis*

Les 2 souches (927 et 531) utilisées ont été isolées de mycétomes mexicains par le Docteur P. Lavalle. Inoculées par voie intrapéritonéale à la souris, elles ont entraîné la formation de très nombreux abcès péritonéaux, spécialement dans la région gastro-splénique. Tous les animaux ont été sacrifiés à la 3 ème ou à la 5 ème semaine, il n'y a eu aucune mort spontanée, à l'inverse de ce que nous avons signalé pour *N. asteroides*.

Les abcès péritonéaux sont toujours riches en grains visibles à l'examen direct (Pl. II, fig. 5). Histologiquement, ces grains ont des formes très variées, souvent très complexes : virgules, accents circonflexes, bandes polymorphes. Toutes ces formes varient en longueur de 70-120 μ; les largeurs oscillent entre 13 et 20 μ. Parfois les grains sont lobés et cartographiques, atteignant alors 300 et même 400 μ. Ils ne sont jamais massués (fig. 2; Pl. II, fig. 6-9; Pl. III, fig. 2-4).

La masse même du grain se colore en rose ou en mauve par l'hématéine-éosine : elle est formée de ponctuations d'affinités tinctoriales variables, soit pour l'hématéine, soit pour l'éosine. Dans les zones où le feutrage est moins dense, on distingue dans un même filament certaines parties roses, avec de courts fragments intercalaires hématéiphiles. Au Ziehl-Gram, dans les grains les plus gros, les filaments sont groupés en couronnes périphériques. Des vésicules,

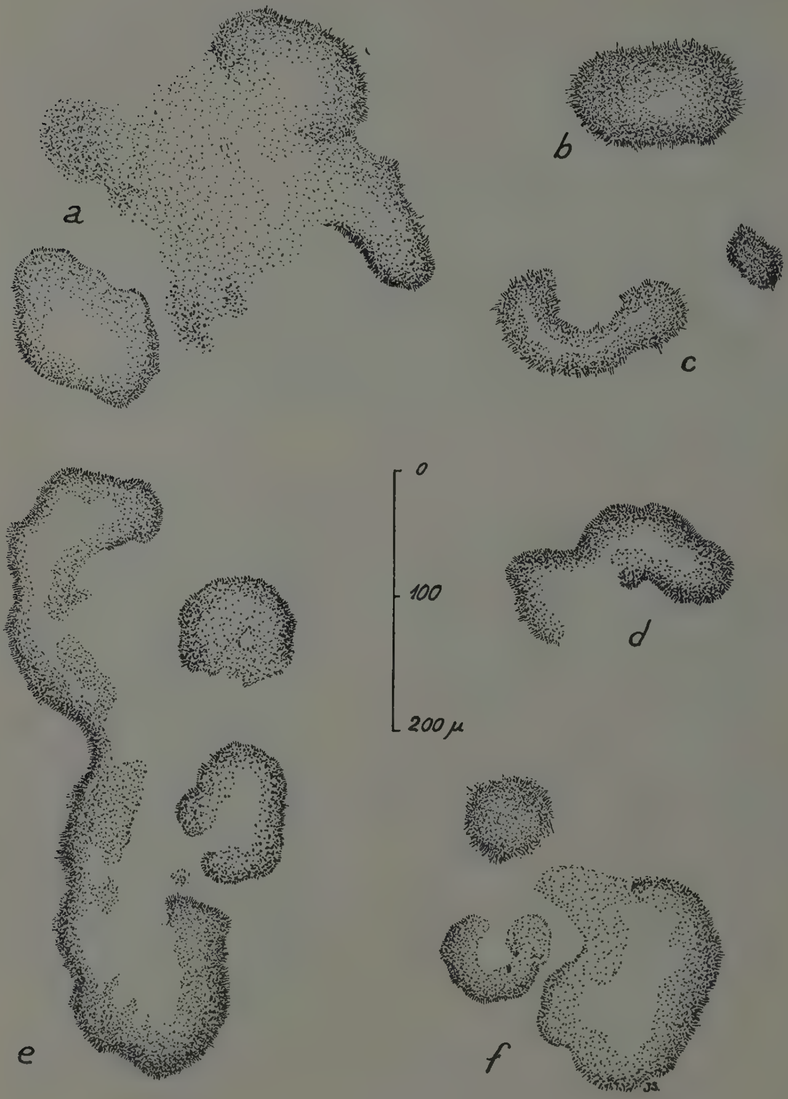

Figure 2.—Diverses images de grains expérimentaux obtenus chez la souris par inoculation de la souche 927 de *N. brasiliensis*. De telles morphologies s'observent fréquemment dans des mycétomes spontanés à *N. brasiliensis*.

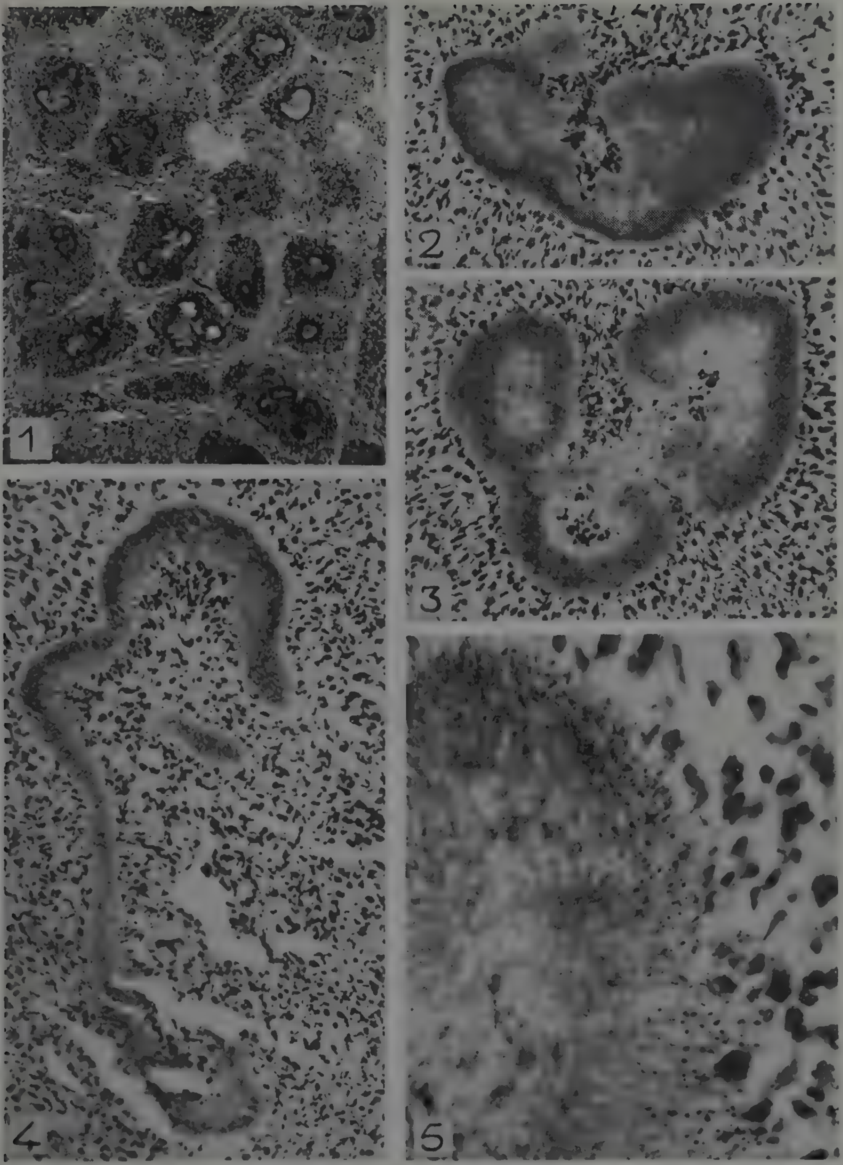

PLANCHE III : Grains expérimentaux

Souris inoculées avec *N. brasiliensis* 927 avec ou sans bacille tuberculeux tué.

Figure 1.—Aspect général de la lésion (*N. brasiliensis* 927 seul) Hémalun. 31 ×.

Figures 2 et 3—Grains lobés obtenus avec *N. brasiliensis* 927 avec bacille tuberculeux tué. Hémalun. 260 ×.

Figure 4.—Grain rubané obtenu avec *N. brasiliensis* 927 seul. Hémalun. 260 ×.

Figure 5.—Bord d'un grain (*N. brasiliensis* 927 seul) coloré au Ziehl-bleu et montrant les ponctuations acido-résistantes sur le trajet des filaments périphériques. 1000 ×.

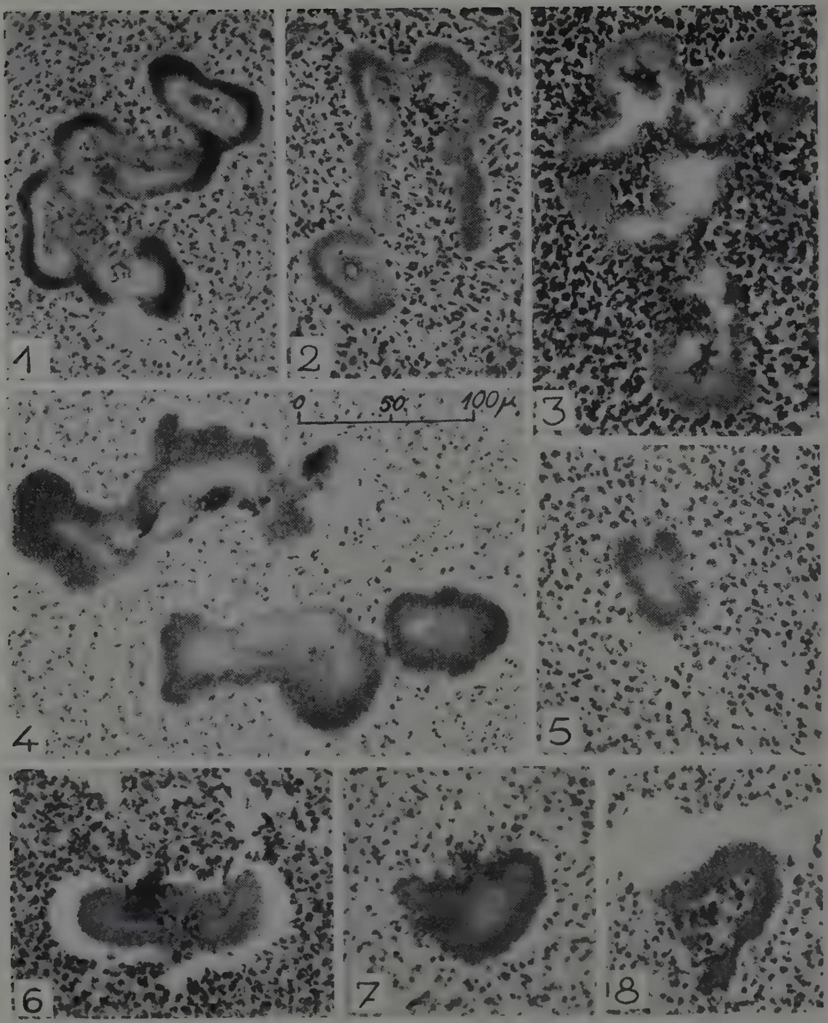

PLANCHE IV : Mycétome d'étiologie inconnue

Figure 1.—Mycétome 1 de Dakar, grain vermiforme finement massué Y 252. Ziehl-bleu. 260 ×.

Figures 2 et 3—Mycétome 2 de Dakar Y 813. Aspect vermiforme (fig. 2) ou polylobé (fig. 3). Hémalun. 260 ×.

Figure 4.—Mycétome de Mogadiscio (Somalia) BA 690. Grain plus volumineux mais structure générale comparable aux précédents. Ziehl-bleu. 260 ×.

Figure 5.—Mycétome du Sud Cameroun (5 ème cas) BB 472. Grains de petite taille, massués. Hémalun. 260 ×.

Figure 6.—Mycétome du Vietnam BA 1263. Hémalun. 260 ×.

Figures 7 et 8—Mycétome de Tahiti Z 661. Hémalun. 260 ×.

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souvent nombreuses et rapprochées, s'observent sur le trajet des filaments. Une nette fragmentation de ces derniers en éléments coccoïdes n'est pas visible. Une acido-résistance partielle a été surtout observée avec la souche 927 (Pl .III, fig. 5).

### III—*Mycétomes d'Etiologie Inconnue*

Les mycétomes à petits grains d'étiologie inconnue étudiés ci-dessous nous ont été adressés d'Afrique, du Vietnam et de Tahiti au cours de ces dernières années.

Notre matériel se compose de :

—2 cas de mycétomes à *Nocardia* venant de Dakar (R. Camain, Institut Pasteur, Dakar).
—1 cas de Djibouti
—1 cas de Mogadiscio adressé par le Docteur Rosati.
—5 cas du Sud Cameroun dont 4 viennent d'être signalés par R. Blaché *et al.*, le cinquième provenant de A. Gamet (Institut Pasteur, Yaoundé).
—1 cas du Vietnam (Docteur Nquyen Van Ai 1960)
—1 cas de Tahiti (Docteur Borries)

Ces 2 derniers sont les premiers cas rapportés d'une telle affection en ces territoires.

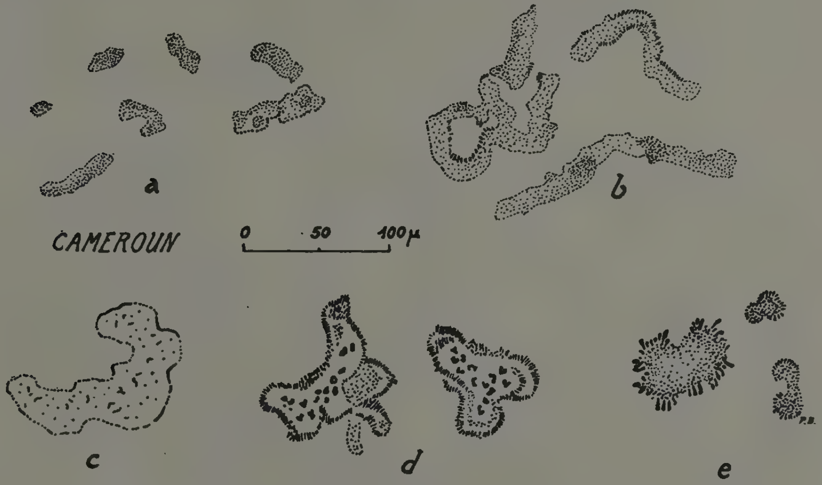

Figure 3.—Morphologies histologiques des grains de 5 mycétomes du Sud Cameroun.

Le planche IV fournit les images histologiques de grains observés dans les abcès de mycétomes provenant de Dakar (Pl. IV, fig. 1-3) ou de Mogadiscio (Pl. IV, fig. 4) de Yaoundé (Pl. IV, fig. 5), de Saigon (Pl. IV, fig. 6) et de Tahiti (Pl. IV, fig. 7, 8). La figure 3 schématise les morphologies constatées dans 5 mycétomes à petits grains camerounais ; enfin, la figure 4 précise certains des grains de Mogadiscio, de Dakar et de Djibouti.

Parfois ces grains restent de petite taille, sont peu étirés et peu massués, tels ceux du Cameroun figurés en *a* de la fig. 3. Ils sont parfaitement identiques aux grains de *N. asteroides* décrits pas Conant. D'autres sont petits, massifs, denses et massués (fig. 3*e*). En *d* les grains montrent un feutrage périphérique dense, finement massué avec des zones centrales envahies de polynucléaires. En *b* le grain s'étire, prend un type vermiforme qui, dans l'espace, doit correspondre à une lame ondulée ou bourgeonnante. Le dessin *c* montre un grain plus trapu, où les

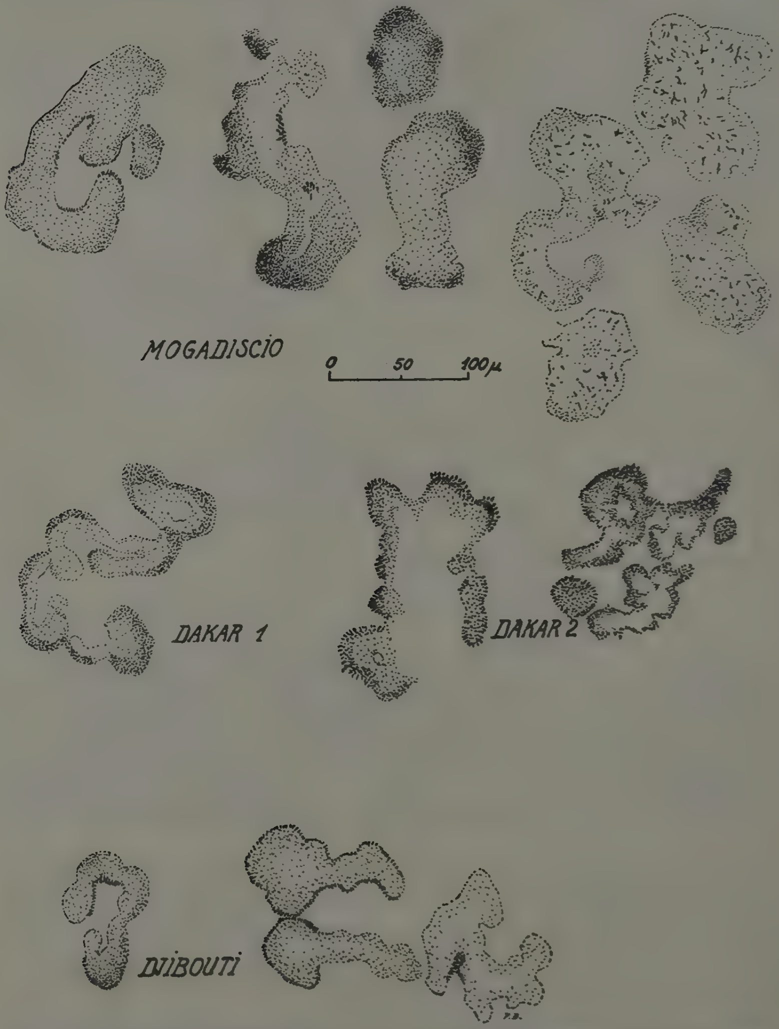

Figure 4.—Morphologies histologiques des grains de mycétomes de Somalia (1 cas), du Sénégal (2 cas) et de Somalie (Djibouti) (1 cas).

filaments sont relativement peu nombreux, dispersés et ne formant pas de feutrage périphérique. Il est assez voisin des 4 grains de Mogadiscio réprésentés à droite de la fig. 4. Mais, dans le même cas de Mogadiscio, s'observent aussi des grains (partie centrale et gauche de la fig. 4) qui se rapprochent de ceux de Dakar 1 et 2 et aussi de ceux de Mexico (Pl. I, fig. 2). Les grains de Djibouti (dessin fig. 4) s'apparentent à ceux décrits par Conant, mais aussi à ceux de Mogadiscio et de Dakar.

Enfin, les grains de Saigon et de Tahiti (Pl. IV, fig. 6-8) sont extrêmement voisins de ceux de Stanleyville (*N. brasiliensis*).

## Discussion

L'étude morphologique des grains de 11 mycétomes à "petits grains" et leur comparaison avec les mycétomes dûs à *N. asteroides* et *N. brasiliensis* permettent de les classer en 4 groupes : dans le premier groupe se rangent les grains trapus, fortement massués (Dakar 2, Cameroun *d* et *e*, Saigon et Tahiti). Le second groupe réunit les grains allongés, vermiformes, irrégulièrement massués (Dakar 1 et 2, Mogadiscio et Cameroun *b*). Il arrive que, dans un même mycétome, des morphologies variées puissent faire hésiter entre l'un ou l'autre des 2 premiers groupes. Cette hésitation est sans importance en fait, puisqu'on peut rapporter ces 2 groupes à *N. brasiliensis*, en raison des images observées dans les cas mexicains et congolais, sûrement diagnostiqués.

Les grains d'un troisième type (Mogadiscio, Djibouti et Cameroun *c*), plus volumineux et massifs, sont formés d'un feutrage lâche, où la technique de Gram ne révèle que d'assez rares filaments actinomycosiques. Ils ne peuvent être actuellement rapportés avec certitude à l'une ou l'autre espèce lorsqu'ils sont seuls présents dans une préparation. Parfois cependant, comme dans le cas de Mogadiscio, ils sont associés à d'autres grains qui, eux, évoquent beaucoup *N. brasiliensis*.

Quant au quatrième type (Cameroun *a*) nous suggérons de l'attribuer à *N. asteroides*.

Dans une faible mesure, l'observation des grains expérimentaux étaye ces suggestions. La morphologie des grains obtenus en inoculant les souches de *N. brasiliensis* est très comparable à celle des grains spontanés. Toutefois, dans ces derniers, des massues sont très souvent présentes, alors que les grains expérimentaux de nos essais ne sont pas massués. Il est vrai que ce peut être là une question de souche ou de conditions expérimentales. Les grains obtenus en inoculant *N. asteroides* sont habituellement différents de ceux du seul mycétome à *N. asteroides* que nous ayons pu examiner. Pourtant, certains grains massués sont superposables à des grains spontanés de *N. brasiliensis*. Une grande prudence est donc à adopter dans l'interprétation des formes obtenues avec l'une ou l'autre espèce.

S'il ne semble pas que jusqu'à présent et avec le peu de souches utilisées, les grains expérimentaux puissent être d'un grand secours dans le diagnostic, en revanche leur analyse peut présenter un intérêt théorique. On retrouve dans les lésions expérimentales à *N. brasiliensis* des types de grains dont les formes dans l'espace et l'évolution sont intéressantes à considérer. On rencontre d'ailleurs ces formes dans quelques mycétomes spontanés dûs à cette même espèce. Il ne s'agit pas là, comme dans les autres mycétomes actinomycosiques, d'un sphéroïde plus ou moins irrégulier, mais de formes infiniment plus complexes. L'examen de coupes en série permet de préciser ces formes: c'est souvent l'aspect d'une membrane qui, au sein de l'abcès, est simplement repliée ou enroulée en cornet, selon un modèle plus ou moins compliqué.

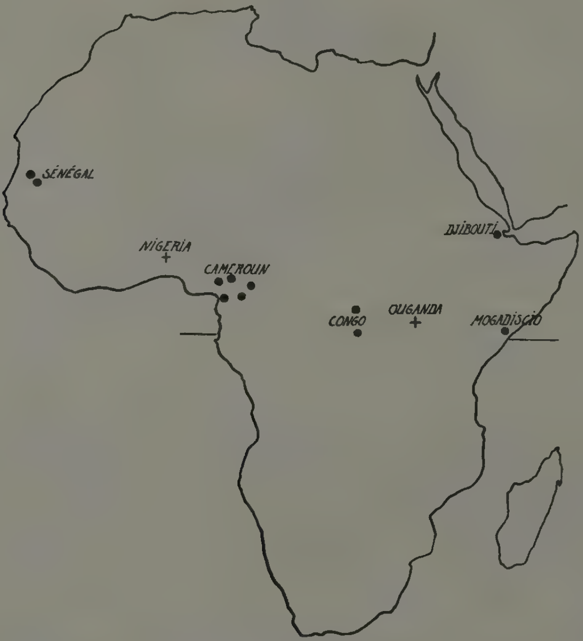

Figure 5.—Répartition géographique des mycétomes africains à *N. brasiliensis* et *N. asteroides*.

La biogéographie des mycétomes à *Nocardia* en Afrique, peut actuellement, en fonction des quelques cas recensés, être esquissée ainsi (fig. 5). Dans la zone d'endémie sahélienne des mycétomes, zône qui s'étend en écharpe du Sénégal aux Pays Somalis, en passant par le Niger, le Tchad et le Soudan, les cas à petits grains sont rares. R. Camain, à l'institut Pasteur de Dakar, sur plus de 100 mycétomes divers, n'en a rencontré que 2 (opérés par O'Connor) attribuables à *Nocardia* sp.* A Djibouti, sur 32 mycétomes, un seul peut être rapporté au genre *Nocardia*. En raison de l'analogie de ses grains avec ceux décrits par Conant (1955),

* M.Rey dans sa thèse de Médecine, actuellement souse presse, fait état de huit cas de mycétomes à "petits grains" observés à l'Institut Pasteur de Dakar (Note ajoutée en cours d'impression).

Destombes, Dutour, Marguet, Mariat & Segretain (1958) l'ont rapproché de *N. asteroides*. De même à Mogadiscio (Somalia), Rosati, parmi les 35 mycétomes (avril 1961) de sa seconde campagne, n'a observé qu'un seul d'entre eux renfermant des petits grains. Abbott (1956) dans les résultats de son enquête au Soudan ne mentionne pas de mycétomes qui puissent être rapportés au genre *Nocardia*. Au Niger, J. Darrigol n'en a pas encore rencontré.

Cette rareté des mycétomes à "petits grains" en zone sèche, domaine essentiel des mycétomes fongiques à *Madurella mycetomi* et *Leptosphaeria senegalensis* et actinomycosiques à *Streptomyces madurae, S. somaliensis* et *S. pelletieri,* met en relief leur relative fréquence dans des territoires équatoriaux très humides. Vanbreuseghem *et. al.* (1956) ont décrit les 2 premiers cas africains de mycétomes à *N. brasiliensis*; ils provenaient de la province orientale du Congo ex-Belge. Blaché *et al* (1961) ont signalé que sur 5 mycétomes étudiés au Sud Cameroun, 4 renfermaient des petits grains. Pour la même région, il s'en ajoute un cinquième qui nous a été communiqué par A. Gamet (Institut Pasteur de Yaoundé). En Nigeria méridionale (Ibadan), Cockshott & Rankin (1960) signalent que parmi les principaux responsables de mycétomes il faut compter *N. brasiliensis*. Enfin, Wilson (1961) au cours d'une enquête dont les résultats ne sont pas encore publiés a observé plusieurs mycétomes à *Nocardia* en Ouganda; il a isolé à plusieurs reprises *N. asteroides* (au moins 5 souches) et *N. brasiliensis* (1 souche).

Jour après jour, les régions du globe où l'on rencontre les mycétomes à *Nocardia* deviennent plus nombreuses et plus étendues. On a longtemps pensé que les mycétomes à *N. brasiliensis* étaient limités à l'Amérique. Ils existent au Brésil et en Uruguay où Mackinnon & Artagaveytia-Allende (1956) ont rapporté 2 cas à *N. asteroides*. En Amérique du Nord, Conant a figuré un mycétome à *N. asteroides*, et Moore, Lane & Gaul (1954) ont décrit un mycétome à *N. brasiliensis*. Ils sont fréquents au Mexique, dans les régions à climat subtropical d'altitude. On les observe maintenant en Afrique. On sait que les mycétomes à "petits grains" existent également ailleurs puisque nous rapportons ici même, un cas observé au Vietnam et un autre à Tahiti. On peut considérer comme probable que ces mycétomes sont plus fréquents qu'il ne paraît au moins dans les zones géographiques où sont réunies les conditions favorables à leur apparition.

De nouvelles recherches combinant l'étude histologique et bactériologique dans un nombre suffisant de mycétomes à 'petits grains' sont donc indispensables. Il serait souhaitable que le seul examen histologique de ces petits grains puisse suffire à distinguer l'espèce en cause, comme c'est le cas pour les autres actinomycètes*. Ce diagnostic histologique nous semble un point capital à préciser, étant donnés l'importance grandissante de cette catégorie de mycétomes, sa répartition géographique ainsi que les problèmes d'ordre épidémiologique et thérapeutique qui y sont liés.

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* Notre travail étant sous presse, nous prenons connaissance du travail de Adela Pizzoli (1961) qui traite du même sujet. Ses conclusions concordent avec les nôtres: il n'est pas possible actuellement de distinguer histologiquement les grains des 2 espèces de *Nocardia*.

## Summary

The authors illustrate the morphology of the "small grain" mycetomas which they have obtained from Africa, Vietnam and from Pacific areas during the past few years. These are rare in the mycetomas of the Sahel region but become prominent in the equatorial zone of Africa. An attempt is made to assess the importance of *Nocardia asteroides* and *N. brasiliensis* and the morphological aspects of grains obtained experimentally with cultures of these 2 species are described.

These investigations indicate the predominant part played by *N. brasiliensis* in the etiology of small grain mycetomas. However, *N. asteroides* also, is a very probable cause of this condition in a certain number of cases. Further investigations are required because of the doubts which still exist and because of the growing importance of this type of mycetoma.

## Remerciements

Nous exprimons notre gratitude aux divers correspondants qui ont bien voulu nous adresser leur matériel ou le résultat de leurs travaux.

Nous remercions également très vivement M. Jacques Grzelak qui a réalisé pour nous de très nombreuses photographies dont seulement quelques-unes illustrent ce mémoire.

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# THE ASCIGEROUS STATE OF *MICROSPORUM COOKEI*

L. Ajello

*Communicable Disease Center, Public Health Service,*
*U.S. Department of Health, Education, and Welfare,*
*Atlanta 22, Georgia.*

Sexual reproduction developed spontaneously in a soil isolate of *Microsporum cookei*. Cleistothecial formation was induced in a second soil isolate by growing it on horse hair on a soil plate. The perfect form of *M. cookei* was classified in the genus *Nannizzia* of Stockdale and given the name of *N. cajetana*. Through the mating of single ascospore cultures of *N. cajetana* with 10 isolates of *M. cookei,* cleistothecia were produced by 5 of the isolates.

*Microsporum cookei* was described as a new dermatophyte in 1959 (Ajello). This fungus is widely distributed in soil and is frequently isolated from rodents and other animals (table 1). Its natural occurrence in soil was disclosed by baiting plates of soil with bits of sterilized human hair (Vanbreuseghem 1952, procedure) and selectively isolating it from that habitat along with other species of keratinophilic fungi.

| *Country* | *Habitat* | *Reference* |
|---|---|---|
| NORTH AMERICA | | |
| *Canada* | | |
| Alberta | Soil | Carmichael, 1959 |
| *United States* | | |
| Florida | Wild animals | Ajello, 1959 |
| Georgia | Wild animals, soil | ,, ,, |
| Idaho | Soil | Cooke, 1952 |
| Kentucky | Soil | McDonough *et al.,* 1961 |
| Michigan | Soil | Ajello, 1959 |
| Pennsylvania | Dog | Ajello, 1959 |
| Washington | Soil | Cooke, 1952 |
| EUROPE | | |
| *Norway* | | |
| Oslo area | Soil | Lindquist, 1960, 1961 |
| *Poland* | | |
| Szczecin area | Soil | Bieluńska, 1961* |
| ASIA | | |
| *India* | | |
| Mysore | Rat | Ajello, Trapido, 1960 |
| AUSTRALASIA | | |
| *Australia* | | |
| Brisbane area | Soil, Rat | Ridley, 1960 |

* Miss Bieluńska observed the development of cleistothecia in one of her isolates of *M. cookei.*

Table 1. Geographic Distribution of Microsporum Cookei.

Study of the development of *M. cookei* on hair revealed that several isolates reproduced by both asexual and sexual means. The purpose of this report is to describe the perfect stage of *M. cookei.*

## OBSERVATIONS

From a group of 519 soil samples collected in the city of Dalton, Georgia in 1959 (Edwards, Ajello, Moore, Jacobs & Aronson, 1960), 11 samples yielded *M. cookei* (unpublished data).

Cleistothecia were first observed on the human hair strands used to bait sample number 295. In August 1959, Mr. P. K. C. Austwick, Central Veterinary Laboratory, Ministry of Agriculture, New Haw, Weybridge, Surrey, England, reported at the Montreal, Canada meeting of the IX International Botanical Congress that Dawson & Gentles (1961) had obtained the perfect stage of *Keratinomyces ajelloi* and other dermatophytes by using horse hair in place of human hair as bait. Accordingly horse hair was used in an attempt to induce sexual reproduction in 14 isolates of *M. cookei* maintained in the laboratory's culture collection (table 2).

| *Code Number* | *Source* |
|---|---|
| A—999 | Soil —Saginaw, Michigan |
| B—140 | Opposum —Newton, Georgia |
| B—275 | Soil —Idaho (one of Cooke's isolates received from Emmons #246) |
| B—276 | Single spore isolate of A—999 |
| B—296 | Soil —Lexington, Kentucky |
| B—359 | Rat —Mysore, India |
| B—157 | Cotton Rat—Newton, Georgia |
| L—69 | Soil —Lexington, Kentucky |
| L—496 | Soil —Lexington, Kentucky |
| L—816 | Soil —Lexington, Kentucky |
| X—36 | Cotton Rat—Newton, Georgia |
| X—202 | Dog —Pennsylvania |
| X—256 | Rat —Australia (received from Mrs. Mary B. Ridley) |
| X—301 | Soil —Szczecin, Poland (received from Miss Sabine Bieluńska) |

TABLE 2. ISOLATES OF *Microsporum cookei* STUDIED.

Top soil was gathered from a wooded area, taken to the laboratory and dispersed in a group of Petri dishes. These plates, along with several others containing horse hair cut into 1 cm. lengths, were sterilized by exposing them to ethylene oxide vapour for 24 hours. This was carried out in an autoclave modified to permit gaseous sterilization. Gas rather than heat was used for sterilization since Gentles (1960) was of the opinion that soil sterilized by heat inhibited the development of perithecia.

About 48 pieces of sterilized horse hair were placed on the surface of the soil plates that had been moistened with sterile distilled water. These were inoculated with small pieces of the stock cultures of *M. cookei,* and incubated at room temperature in a cupboard for several weeks. The plates were examined periodically for the development of cleistothecia on the surface of the sporulating mycelium that covered the hair and surrounding soil. Under these cultural conditions, only Kentucky soil isolate L69 developed cleistothecia.

The cleistothecia appeared as small, ivory spheres on the mycelium that overgrew the hair (Fig. 1 a, b). These could be picked up individually with a needle and studied under the microscope (Fig. 1c). Observation was facilitated by placing the cleistothecia in a drop of 95 % ethyl alcohol prior to mounting in lacto-phenol cotton blue. This served to eliminate air bubbles.

The cleistothecia of *M. cookei* possessed the general characteristics of the genus *Nannizzia,* recently described by Stockdale (1961). The peridium of the cleistothecia was composed of interwoven, hyaline, septate, branched hyphae. These thin-walled filaments were echinulate and slightly constricted at the point of septation (Fig. 1 d, e). Appendages, in the form of elongated, septate, smooth mycelial filaments (Fig. 1 e), and coiled mycelium (Fig. 1 f) extended from the peridial mycelium. When crushed, thin-walled, hyaline, globose asci—that contained 8 golden ascospores—escaped from the cleistothecia (Fig. 2 a).

Various stages in the development of the cleistothecia were observed on the hair filaments (Fig. 2 b-d). The perithecial initials were in the form of a clavate antheridium and a filamentous ascogonium that coiled around the antheridium. These initials became envoloped by peridial hyphae. Evolution of the cleistothecia essentially follows the sequence described by Stockdale (1961).

Several cleistothecia of this isolate of *M. cookei* (L69) were airmailed to Miss Christine Dawson, Department of Bacteriology, University of Glasgow, who prepared single ascospore isolates. Through the crossing of these isolates, she and Dr. J. Gentles were able to demonstrate that the ascigerous stage of *M. cookei* is heterothallic.

The perfect stage of *M. cookei* has been named *Nannizzia cajetana* in honour of my father, Gaetano Ajello.

**Nannizzia cajetana** sp. nov.

Fungus heterothallicus. Cleistothecia globosa, color eboris, 368-686μ C. Hyphae peridii hyalinae, septatae, verticillatim ramosae. Cellae dense asperulatae, cum parietibus tenuibus, leviter constricti ad situ septatarum. Hyphae apicibus libiris numerosis, appendiculis biformibus: (1) hyphae elongatae, graciles, parietibus laevibus, usque ad 480μ de longitudiine, ab 3·6μ ad basem, 2·4μ ad centrum, et 1·2μ ad apicem; (2) hyphae elongatae, gracilis, parietibus laevibus, strictim volutae. Asci globosi, vel ovati, 6-9μ D, octospori. Ascospori ovati, lueti, parietibus laevibus, 3-3·6μ × 1·8μ.

*Stat. conid. Microsporum cookei* Ajello, (1959).

*Habitatio* : naturalis solum, cornuaffinitiva.

Heterothallic. Cleistothecia globose, pale yellow, 368-686μ D. Peridial hyphae hyaline, septate, verticillately branched. Mycelial cells echinulate, thin-walled and slightly constricted at site of septations. Free ends of peridial hyphae numerous with two kinds of appendages : (1) elongated, slender, tapered, smooth hyphae up to 480μ in length, 3·6μ in D at base, 2·4μ at mid-length and 1·2μ at tip; (2) elongate, smooth-walled slender hyphae coiled into spirals.

Asci globose or ovate, 6-9μ D, 8-spored. Ascospores ovate, smooth-walled and golden, 3-3·6μ × 1·8μ.

*Conidial stage* : *Microsporum cookei* Ajello, 1959.

*Habitat* : Soil, keratinophilic.

The type culture of *N. cajetana* is ATCC #14386. This culture (L69) was recovered from soil collected in the Lexington, Kentucky area and is designated as B433 in the CDC fungus collection. The "+" and "–" phases of *N. cajetana* bear the ATCC numbers of ATCC #14387 (+), and ATCC #14388 (–). They were prepared by Miss C. O. Dawson from the type culture.

## DISCUSSION

The "+" and "–" single ascospore isolates of *N. cajetana* (L69) received from Miss Dawson were used to cross with 10 stock culture isolates of *M. cookei*. Cleistothecia were formed in 5 of these crosses (table 3). The basis for the failure of 5 strains of *M. cookei* to produce cleistothecia is unknown. A similar situation was encountered by Stockdale when she mated cultures of *M. gypseum* with single ascospore isolates of *N. incurvata*. She stated that this failure suggested "that *M. gypseum* may be the imperfect state of more than one species". This is an untenable hypothesis, since an imperfect species has never been shown to possess more than a single perfect state.

| *M. cookei X* | *L69+* | *L69–* |
|---|---|---|
| A—999 | Cleistothecia | — |
| B—140 | Cleistothecia | — |
| B—157 | — | — |
| B—275 | — | — |
| B—276 | Cleistothecia | — |
| B—296 | Cleistothecia | — |
| B—359 | — | — |
| X—36 | — | — |
| X—202 | — | Cleistothecia |
| X—301 | — | — |

TABLE 3. MATING OF *M. cookei* WITH "+" AND "–" ISOLATES OF *N. cajetana* (L69).

Since the rediscovery of sexual reproduction in *M. gypseum* by Stockdale (1961), the perfect stage of *Microsporum nanum* (*N. obtusa*) and of *Keratinomyces ajelloi* (*Arthroderma uncinatum*) have been described by Dawson & Gentles (1961). I have observed abortive ascocarps in several cultures of *Trichophyton mentagrophytes*, that resemble those of the genus *Arthroderma*. On the basis of these findings and observations, the dermatophytes can no longer be considered to represent a degenerate group of molds that in the course of their evolution as parasites have lost their ability to reproduce sexually. In due time the conditions necessary to induce sexual reproduction in many, if not all, of the dermatophytes will probably be discovered. Such an achievement will permit the classification of the dermatophytes and the evaluation of species validity on the basis of firm botanical criteria.

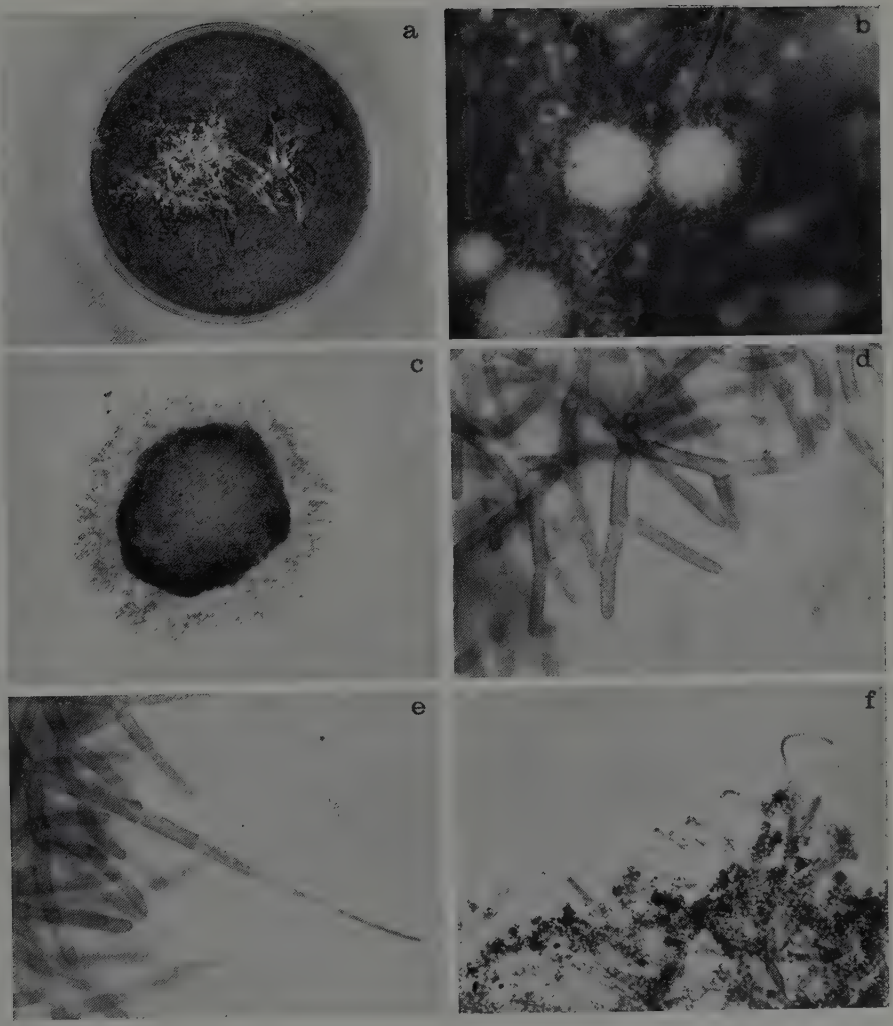

FIGURE 1 *Nannizzia cajetana*

a. Cleistothecia growing on horse hair in a 4-week-old soil plate.
b. Surface view of ascocarps photographed with epi-illumination ×45.
c. Whole mount of a cleistothecium ×60.
d. Echinulate peridial hyphae ×600.
e. Long, tapered projection from peridial hypha ×600.
f. Coiled projection from peridium ×360.

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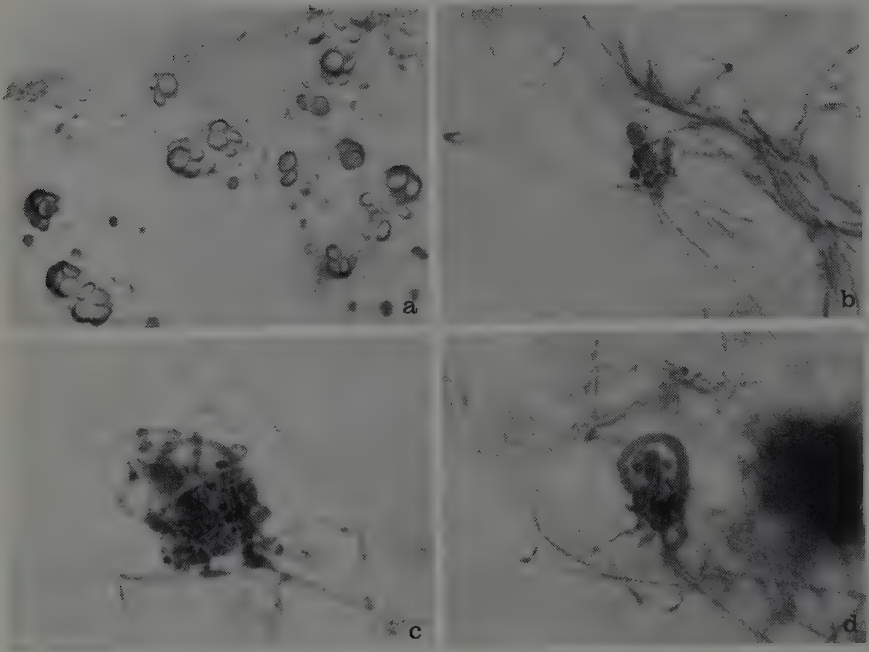

FIGURE 2. *Nannizzia cajetana*

a. Asci and ascospores ×1500.

b, c, d. Clavate antheridia surrounded by coiled ascogonia, ×600, ×900, ×600.

## Résumé

Une reproduction sexuée spontanée est observée dans une culture de *Microsporum cookei* isolée de sol. Dans un second échantillon de sol cultivé sur crin de cheval on obtient la formation de cleistothèces. La forme parfaite de *M. cookei* a été classée dans le genre *Nannizzia* de Stockdale et on lui a donné le nom de *N. cajetana*. Par la copulation de cultures monoascosporées de *N. cajetana* avec 10 souches de *M. cookei,* la production de cleistothèces est obtenue dans 5 cas.

## Acknowledgements

I wish to express my thanks to Miss Christine Dawson for preparing the single ascospore isolates of *N. cajetana*. I also am indebted to Mr. Warren Laing Armstrong, CDC Technical Reports and Library Section, for preparation of the Latin diagnosis and to Mr. John P. Gust, Jr. for the photographic work.

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# *TRICHOPHYTON MENTAGROPHYTES* IN MICE: INFECTIONS OF HUMANS AND INCIDENCE AMONGST LABORATORY ANIMALS

D. W. R. Mackenzie

*Department of Microbiology, Queen's University of Belfast*

Seven children infected with *T. mentagrophytes* had apparently acquired infection from pet mice, all of which had been bought at the same shop. Examination showed that 12 out of 20 animals tested were carrying infection. Examination of laboratory breeding boxes showed that in 49 out of 160 (30%) the mice were harbouring infection; in stock cages the fungus was isolated from 104 out of 147 (70%) individual mice. Two adult laboratory workers routinely handling these mice became infected. Symptoms were observed in only 2 mice out of the 104 shown to carry infection. The methods did not distinguish between true and apparent infections of the mice.

Between February and April 1961, 7 children (5 boys and 2 girls, ranging from 7-12 years) examined at the skin clinics of the Royal Belfast Hospital for Sick Children, and the Ards Hospital, Newtownards, Co. Down, had ringworm infections (caused by *T. mentagrophytes*) attributed to contact with pet mice. In most cases, confirmation by mycological examination of the animals was not possible, and the implication of the pets as sources of infection was largely presumptive. On 1 occasion, however, the mouse belonging to 2 infected brothers was examined in the laboratory and shown to be infected. In another family, 2 sisters and a brother were infected, but the pet mouse was not obtained for mycological examination in this or the remaining families.

In addition to these 7 children, 2 technicians in this laboratory (male and female of 26 and 23 years of age respectively) who handle mice acquired infections of the glabrous skin caused by *T. mentagrophytes*.

When it became known that pet mice were a possible source of ringworm, attempts were made to locate their origin, and to determine if infected animals were being sold at pet shops in Belfast. At the same time studies on the occurrence and incidence of *T. mentagrophytes* were made in the mouse population of this laboratory.

## METHODS

(a) Direct examination: All mice were examined for the presence of lesions, particular attention being paid to the presence of localised or diffuse hair loss, scaling and/or broken hairs.

(b) Direct swabbing: Animals were held by the tail and a bacteriological swab was rubbed over the abdomen, back, neck, face and tail. In examining animals at the pet shop, petri dishes containing 10-12 ml. 2·5% malt agar incorporating antibiotics (penicillin 20 units/ml.; streptomycin 40 units/ml.; cycloheximide 0·5 mgm./ml.) were inoculated and incubated for 7 days at 26° C. Colonies of *T. mentagrophytes* appeared within 3-4 days. Animal cages were examined by the same method. Swabbing by means of sterile gauze pads as described by Mackenzie (1961) was originally employed, but discarded in favour of bacteriological swabs. In examining laboratory mice, bacteriological swabs were used throughout to inoculate the surface of approximately 1·5 ml. of the selective medium contained in small (5 ml.) screw top bottles.

(c) Sampling without intervention of swabs: In 1 experiment described below, infected mice were placed directly on the surface of the malt agar contained in a large (36×30 cm.) stainless steel tray.

## RESULTS

*Pet shop*

Of 9 pet shops visited, 6 did not sell mice; of the remainder, only 1 had a stock of mice during the period of investigations. Not surprisingly, this was the shop from which all infected children had bought their pet mice. A request was made to the owner of this shop to examine the stock, and permission was granted to make a limited number of samples. At that time the stock consisted of approximately 50 mice, in 2 stock cages each containing 20 mice, and 1 breeding box containing 2 adults and a litter of newborn animals.

*T. mentagrophytes* was isolated from 8 out of 11 animals tested in 1 stock cage. It was also isolated from the 2 adult mice in the breeding cage. Ten swabs of the walls of cages and boxes were all negative.

*Laboratory mice*

During the period of investigations, the combined breeding and stock population totalled 780 weaned mice. Unweaned animals were not selected for study. Four hundred and eighty mice (160 male, 320 female) were housed in 160 breeding-boxes, each box containing 1 male and 2 females. Individual breeding boxes rather than individual mice were examined, and a single swab was used on the 3 adult mice within each box.

| *No. of cages* | *No. of mice* | *No. of boxes positive* | *% of boxes positive* |
|---|---|---|---|
| 160 | 480 | 49 | 30·6 |

TABLE 1.—EXAMINATION OF BREEDING BOXES FOR PRESENCE OF *T. mentagrophytes*.

The remaining 300 animals were segregated into 11 stock cages, containing up to 54 male or female mice. All the laboratory mice were inbred albino strains, the animals originally derived from the VSBS strain accounting for all but 29 mice (16 male, 13 female) of an A2G strain. The results are shown in tables 1 and 2.

Only 2 of the 104 animals shown to be carrying infection showed symptoms suggestive of ringworm. Both mice had areas of diffuse hair loss on the trunk; circumscribed lesions were not seen. Many of the mice examined presented a similar appearance, but did not yield *T. mentagrophytes* on sampling. Unequivocal evidence of infection was not observed in either the pet shop or laboratory mice, a total of over 800 animals examined.

| *Cage* | *Strain* | *Male/ Female* | *No. of mice* | *No. tested* | *No. positive* | *% positive* |
|---|---|---|---|---|---|---|
| A | VSBS | Female | 22 | 11 | 10 | 90·1 |
| C | ,, | ,, | 27 | 14 | 12 | 85·7 |
| D | ,, | ,, | 31 | 16 | 16 | 100· |
| E | ,, | ,, | 54 | 23 | 13 | 56·5 |
| G | ,, | ,, | 28 | 13 | 9 | 69·2 |
| L | A2G | ,, | 13 | 7 | 1 | 14·3 |
| B | VSBS | Male | 17 | 9 | 2 | 22·2 |
| F | ,, | ,, | 54 | 28 | 25 | 89·3 |
| H | ,, | ,, | 28 | 14 | 13 | 92·9 |
| J | A2G | ,, | 16 | 7 | 0 | 0 |
| K | VSBS | ,, | 10 | 5 | 3 | 60· |
| Total | | | 300 | 147 | 104 | 70·7 |

TABLE 2.—EXAMINATION OF STOCK CAGES FOR PRESENCE OF *T. mentagrophytes*

During examination of the stock cages, 2 settling plates were exposed for 30 minutes on the floor of the mouse room immediately below the battery of cages. On 1 plate a single colony of *T. mentagrophytes* developed.

### *Direct sampling*

Two white mice were purchased from the pet shop, and although symptoms were absent, swabbing showed that both harboured considerable quantities of *T. mentagrophytes*. Each animal was placed on the surface of 500 ml. of selective medium contained in a large stainless steel tray. The mice were confined to separate halves of the surface by a cardboard partition (fig. 1). After 10 minutes, the mice were removed and the tray incubated at 26° C. After 14 days, numerous colonies of *T. mentagrophytes* were visible on both surfaces (fig. 2).

## DISCUSSION

Infections of humans with *T. mentagrophytes* from mice have been reported on several occasions, particularly where individuals were routinely handling experimental animals. Accidental infections of laboratory personnel have occasionally been followed by mycological examination of animal sources, and the discovery of previously unsuspected reservoirs of infection. Simultaneous infections of laboratory workers and mice have been described by Parish & Craddock (1931), Booth (1952), and Rieth, Schirren, El-Fiki & Ito (1960), whilst the isolation of *T. mentagrophytes* from mice alone has been reported by Dubois (1929), Catanei (1942), Georg, Hand & Menges (1956), Menges & Georg (1957) and Menges, Love, Smith & Georg (1957).

The present observations show that the incidence of naturally occurring *T. mentagrophytes* amongst the mice examined was high, particularly, and least expectedly, amongst the laboratory animals. That the pet shop constituted a potential public health hazard was shown by the demonstration of the fungus on 12 out of 20 mice examined. Two of these mice were shown to deposit considerable quantities of infective material on an agar surface (fig. 2). There is little difficulty in associating the deposition of similar quantities of fungal inoculum on the surface of human skin from such mice with the ultimate appearance of

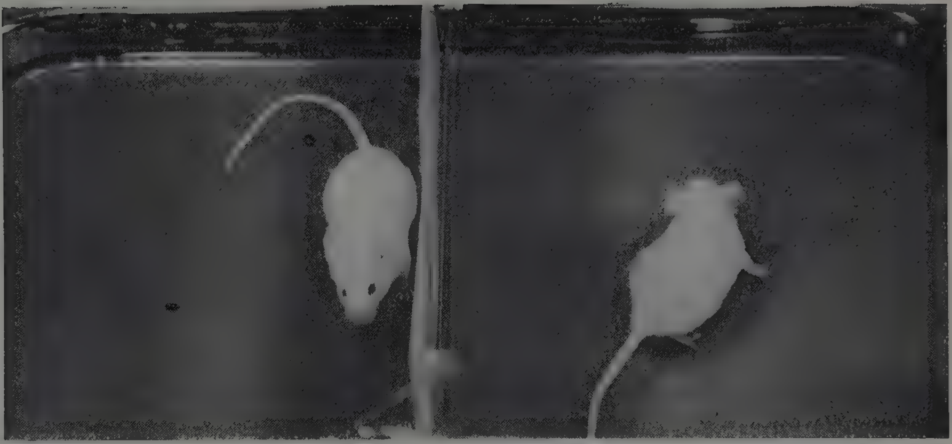

Figure 1.—Infected mice on surface of actidione agar.

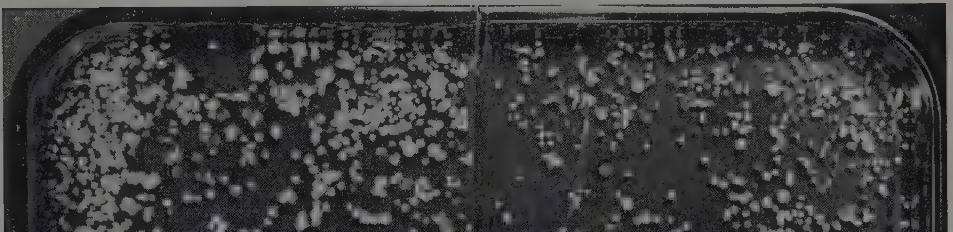

Figure 2.—14 days later. Numerous colonies of *T. mentagrophytes* deposited from infected mice.

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ringworm lesions on the necks, shoulders and arms of the 7 children. Parental suspicion that infection had been acquired from pet mice was usually responsible for the immediate destruction of the animal, and by the time the child was seen at the skin clinic no possibility remained of establishing a link between mouse and child. However, presumptive evidence is strong, and there can be little doubt that all 7 children acquired their infections from pet mice. In view of the amount of infective material shown to be present in the laboratory animals, it is reasonable to suspect that the 2 laboratory workers also acquired infection from mice.

Of especial interest is the marked absence of symptoms amongst the mice. This has been reported by Dolan, Kligman, Kobylinski & Motsavage (1958) who recorded the infection of 3 laboratory workers handling white mice and rats, and demonstrated by cultural techniques that 18% of 'healthy' mice harboured *T. mentagrophytes*. LaTouche (1957) recorded infections in 11 mouse attendants but was able to demonstrate the presence of infection amongst laboratory mice on only 1 occasion, describing symptoms which included hair loss and scaling. Gentles & O'Sullivan (1957) reported the infection of a boy from his pet mouse, the latter showing cultural but not clinical evidence of infection. Lonsdale (1958) has also described the isolation of *T. mentagrophytes* from apparently healthy mice in laboratories and pet shops. It is clear that visible lesions are rarely encountered in natural infections of mice with *T. mentagrophytes*. With 2 possible exceptions, animals examined in the pet shop and laboratory were free from symptoms, and the high proportion harbouring the fungus was revealed only by sampling techniques.

In this study it is important to realise the distinction between 'true' and 'apparent' infections. In the former, although symptoms may be trivial, there has nevertheless been invasion of host tissue and proliferation of the fungus, and the animal will presumably remain infectious as long as fresh arthrospores are being produced. "Apparent" infections are those where infective material deposited from an active lesion (or transferred from a carrier) adheres passively on the coat or skin of a healthy animal. Such an animal could initially constitute as effective a source of infection as one with a 'true' infection, but as fresh fungal material is not being produced, its ability to initiate infection would decrease with time. A single infected mouse in a breeding or stock cage will probably contaminate most, if not all of the other mice, and this would account for the high overall incidence that was obtained. Most of the sampling methods were qualitative and did not distinguish between true and apparent infections. Consequently, the results showed only the number of mice harbouring infection and the true incidence of infection remains unknown. Nevertheless, the amount of infective material present in the laboratory mice was shown to be considerable and any attendant handling the mice would run the risk of acquiring infection.

It is perhaps significant that the only stock cage where infection was not found contained males of a strain of mice (A2G) only recently acquired by the laboratory. Only 1 female animal of this strain was shown to harbour the fungus. It would appear that this mouse was originally uninfected but became so following handling by the attendants. Aerial dispersal of the fungus was also demonstrated and could possibly be considered as an accessory method of dissemination.

## Résumé

Ayant constaté que sept enfants atteints de trichophytie à *T. mentagrophytes* s'étaient infectés par des souris blanches achetées au même magasin, on examina dans cet établissement vingt souris prises au hasard: 12 étaient infectées. Des contrôles révélèrent que 49 sur 160 animaux (30%) étaient infectés dans les boites utilisées pour l'accouplement et la reproduction, et 104 sur 147 (70%) dans les boites où les souris étaient stockées. Deux travailleurs adultes manipulant ces animaux au laboratoire furent atteints. Sur les 104 souris infectées, deux seulement présentaient des symptomes visibles: les méthodes d'examen utilisées ne permettaient pas de distinguer les souris réellement infectées de celles hébergeant seulement le parasite.

## Acknowledgements

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# REVIEWS

*Micosis Profundas* (*Cutáneas y Viscerales*) *Volumen II HISTOPLASMOSIS, Histoplasmosis en la Argentina* (Deep Mycoses (Cutaneous and Visceral) Volume II HISTOPLASMOSIS, Histoplasmosis in the Argentine). PABLO NEGRONI. (Comisión de Investigación Científica, Provincia de Buenos Aires, Gobernación, Publicación Especial N°. 1, La Plata, 1960). Pp. 143, 34 figs. Paper bound.

As the title indicates, this book is part of a series but stands by itself as a comprehensive treatise on histoplasmosis; it is the only textbook on this disease to date. That it has taken so long for a volume of this type to emerge is surprising: no other systemic mycosis involves as large and heterogeneous a sector of the human race or is as widespread geographically, or is as clinically protean, and difficult to prevent. Although two symposium volumes and numerous scattered papers on this disease have been published, they lack the logical sequence and uniformity of treatment of this one.

The task could hardly have been undertaken by anyone better qualified than Dr. Pablo Negroni, mycologist and clinician of the Argentine whose competence and versatility have been known to the medico-mycological world for many years. Dr. Negroni's first-hand experience with a wide variety of clinical types of histoplasmosis, with their treatment (extending over a few decades of therapeutic fashion), with the isolation and study of their causative agent, and with the preparation and testing of antigens for the study of their immunologic reactions are evident throughout the text—such is its richness, clarity, and authenticity of detail. Following a thorough historical review, chapters dealing with the etiologic fungus *Histoplasma capsulatum*; human symptomatology and pathology of the disease; animal pathogenicity; and finally, the characteristics and management of 51 cases of histoplasmosis seen in the Argentine complete its contents, which in addition to tables, fair-to-good illustrations and an extensive, 12-page bibliography, make the book extremely valuable reading to all students of medical mycology, veterans as well as beginners.

The text is in Spanish, an obvious limitation for many workers in North American and African endemic areas, for whom it would be particularly useful. Translation into other languages is urged.

MARGARITA SILVA

*Manual de Micologia Medica* (Manual of Medical Mycology). LACAZ, CARLOS DA SILVA (Sao Paulo. Livraria Atheneu, 1960) 3rd Ed., Pp. 559, 270 figs.

In this third edition of his textbook Professor Lacaz has improved the quality, coverage and usefulness of the Manual. The book is printed on good quality paper and the many illustrations, almost without exception, are clearly reproduced. Several figures in colour illustrate the aspects of cutaneous lesions or of fungi demonstrated in tissue by special stains. Appropriate material is presented in numerous useful tables which are conveniently located but are not numbered.

The first four chapters are general in nature and supply an introduction to mycology, define terms and discuss classification. They review the morphology, biology and habitats of fungi; discuss medical mycology in terms of general features of the mycoses, susceptibility of the host, sources of infection and classification; and describe methods of isolation of fungi, culture media and industrial uses of fungi.

Chapter V describes the superficial mycoses, Chapter VI the deep mycoses and the mycoses are reconsidered from the viewpoint of anatomical location in another short chapter.

Additional chapters are devoted to veterinary mycology, fungal allergens, histopathology, therapy and the technics used in the study of fungi and the diagnosis of mycoses. The book concludes with a glossary, a bibliography and a short index.

Except in the actinomycetes the nomenclature adopted corresponds to that generally accepted. Corresponding to its importance in Brazil, 53 pages are devoted to South American blastomycosis and *Paracoccidioides brasiliensis*. Keloidal blastomycosis is described as a separate, distinct mycosis. Regrettably, the section on histoplasmosis includes the distribution map of Furcolow & Grayson which erroneously omits the area of high frequency of histoplasmin sensitivity in eastern United States. Chromoblastomycosis and sporotrichosis together occupy 37 pages, indicating the importance of these mycoses in Brazil.

Lacaz has presented a solid and authoritative book which the student will find useful for its convenient discussion of technics, media and therapy, as well as for its well illustrated descriptions of specific mycoses.

CHESTER W. EMMONS

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*Studies on Candidiasis in Japan*. Research Committee of Candidiasis (Education Ministry of Japan, 1961). Pp. 200.

In 1955, due to the increasing incidence of candidiasis in Japan a "Research Committee of Candidiasis" was founded in an attempt to clarify aspects of pathogenesis, diagnosis and treatment of this disease. This collection of 20 papers illustrates the scope of the work undertaken and covers the incidence, diagnosis, pathogenesis, pathology and therapy of candidiasis and also mycological and serological studies on the genus *Candida*. Papers on *C. albicans* deal with certain aspects of biology, antigenic preparation and structure, the resistance to trichomycin and the effect of various drugs on the respiration of the fungus. It is regrettable that some of the authors have omitted references to the relevant literature but the work is nevertheless of a high standard and consititutes a valuable contribution to the knowledge of this ubiquitous and troublesome disease.

CHRISTINE O. DAWSON

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*Biologichni vŭprosi na mikologiyata* (Biological problems of mycology). BALABANOV, V.A. (Sofia, State Publishers "Meditsina i fizkultura", 1960). Pp. 164. 81 fig. 4 col. pl.

This little book, in Russian with a 6-page English summary, begins with a brief introduction to moulds and their classification which is followed by a more detailed consideration of the Dermatophyta, in the four "series" of which (Ctenomyces, Sabouraudites, Epidermophyton, and Allanoides (Bodinia) ) a number of species are arranged, Sabouraud's nomenclature being mainly adopted. The relationship of dermatophytes to the skin is discussed and the transition from saprophytism to parasitism is considered. Other chapters deal briefly with *Candida* infections and with systemic mycoses.

*Mikotoksikozȳ cheloveka i sel'skokhozyaĭstvennȳkh zhivotnȳkh* (Mycotoxicoses of man and farm animals) .BILAĬ, V. I., (Kiev, Publishers of the Ukrainian Acad. Sci., 1960). Pp. 168, 22 fig, 10 pl., 5 graphs. Roubles 8.55.

This collection of twenty papers is divided into three groups: "Characteristics and some properties of toxic fungi" (pp. 5-70), "Mycotoxicoses of man" (pp. 71-123), and "Mycotoxicoses of farm animals" (pp. 124-160), and concludes with a 6-page bibliography. Special attention is given to disorders caused by *Fusarium,* especially *F. sporotrichiella, Stachybotrys,* and *Claviceps.* The volume is a useful supplement to SARKISOV's *Mycotoxicoses (fungal poisonings)*, 1954.

Centraalbureau voor Schimmelcultures, Baarn, Netherlands, List of Cultures 26th Edition, 1961.

The list of cultures maintained at the Centraalbureau voor Schimmelcultures under the directorship of Agathe L. Van Beverwijk is now available. This is probably the largest and best known collection of fungi in the world and comprises more than 10,000 strains. The catalogue is divided into 3 parts, Filamentous Fungi, Yeasts, and Actinomycetes, and there is also a supplement of recent acquisitions. In a foreword notes are given of the organization of the Centraalbureau, and of the scientific staff and their recent publications. Dr. G. A. de Vries is in charge of the medical mycolgy section.

Colloque sur les Phénomènes d'immunité en Parasitologie. *Pathologie-Biologie,* 1960, Vol. 8, No. 3-4, Pp. 289-296.

This publication presents the proceedings of a joint meeting of the French Society of Medical Biology and the French Society of Medical Mycology held in Marseille in May 1959.

The subject of the meeting was "The Phenomena of Immunity in Parasitology" and the papers which were presented covered the mycoses generally, and cryptococcosis, dermatophytosis and candidiasis in particular. Recent developments in this field of study and the problems connected with the use of immunity phenomena in diagnosis of mycotic infection are thoroughly dealt with.

## Symposium on Medical Mycology

A Symposium on "The Ecology of the Dermatophytes in Australia and New Zealand" took place during the Thirty-fifth Congress of the Australian and New Zealand Association for the Advancement of Science, held at Brisbane, Queensland from 29th May to 2nd June, 1961. The Symposium, which formed part of the programme of Section I (Microbiology, Epidemiology and Experimental Medicine) took place on Thursday, 1st June at 2.15 p.m. under the chairmanship of Dr. E. Beatrix Durie. Dr. Ian MacKerras, Director of the Queensland Institute of Medical Research presided during the reading of Dr. Durie's Paper.

### *Keratinophilic Fungi in Soil*

A paper on "The Presence of Dermatophytes and Other Keratinophilic Fungi in Soil" by E. Beatrix Durie & Dorothea Frey of the Institute of Medical Research, Royal North Shore Hospital, Sydney was read by Dr. Durie. She said that during the past few decades, a great interest in medical mycology had developed throughout the world. Studies of epidemiology, ecology and taxonomy had occurred so rapidly that the whole subject had become infinitely more interesting and alive. Some of the most interesting of these recent developments concerned the dermatophytes. One of these had begun in 1951 when Emmons and, independently of him, Vanbreuseghem & Van Brussel had pointed to the soil as a reservoir of pathogenic fungi. Since that time, *Microsporum gypseum*, as well as other keratinophilic fungi had been isolated from the soil in Britain, Hungary, Germany, Australia, New Zealand and other parts of the world by the use of soil plates baited with autoclaved hair. From February 1955 to December 1960, Dr. Frey and Dr. Durie had examined 712 samples of soil from various parts of Australia for the presence of keratinophilic fungi. *M. gypseum* had been isolated from 10·4%; *Keratinomyces ajelloi* from 9·4%; *Trichophyton terrestre* from 2·6%; *Aleurisma keratinophilum* (a new species) from 1·7%; *Ctenomyces serratus* Eidam from 1·4%; and *Trichophyton* species from 6·2%. Dr. Durie referred to the work of Dr. Griffin who had studied the total colonization of sterile hair in contact with soil. He had been able to demonstrate a characteristic succession, the initial colonizers being common soil fungi of high saprophytic ability, the final ones slow-growing keratinophilic fungi. By far the most interesting development that had arisen out of the study of hair in contact with soil was the finding of the perfect stage of several dermatophytes. In 1959, Dawson & Gentles had discovered the perfect stage of *K. ajelloi*; Stockdale had identified the perfect stage of *M. gypseum*, naming it *Nannizzia incurvata* after the Italian scientist who had described the perfect stage of *M. gypseum* in 1927, but whose work had not been accepted at that time. It was possible that *M. gypseum* was the imperfect stage of at least 2 perfect fungi. Griffin had confirmed Nannizzi's report of *Gymnoascus gypseum* as a perfect state and Szathmary & Herpay in Hungary, had also rediscovered Nanizzi's cleistothecial fungus. Dawson & Gentles had also found the perfect stage of *T. terrestre* and that of *M. nanum*.

The search for keratinophilic fungi in soil had led to the discovery of a reservoir of infection in ringworm and allied infections; to a wider and less anthropocentric conception of the place of the keratinophilic fungi in nature; to a study of the total characteristic fungal flora and succession in soil baited with hair, and finally to the discovery of the perfect states of several of the Fungi Imperfecti.

### *Trichophyton species from Hedgehogs and Possums*

A paper on "The Isolation of Trichophyton species from Hedgehogs and Possums in New Zealand" by Associate Professor Mary Marples, Department of Microbiology, University of Otago, Dunedin, New Zealand was read by Professor J. A. R. Miles, of the same Department. A survey of 132 non-domestic animals for the carriage of dermatophytes had been undertaken. The sample included 49 hedgehogs (*Erinaceus europaeus*) and from these animals 18 isolates of *T. mentagrophytes* and 5 of *T. terrestre* had been obtained. The main site of infection was at the base of the quill, and many of the animals had very extensive infections. One strain of *T. mentagrophytes* was isolated from the examination of 34 Australian possums (*Trichosurus vulpecula*).

*Dermatophytes in Queensland*

Mrs. M. F. Ridley, from the Queensland Institute of Medical Research read a paper on "The Occurrence of Dermatophytes in Queensland".* A total of 434 dermatophytes had been isolated in the laboratories of the Brisbane Hospitals, the Mater Hospitals, the Queensland Institute of Medical Research and the Commonwealth Health Laboratory, Townsville, from February, 1957 to November, 1960. The species isolated and the ages and site of infection were listed and discussed.

*The Endothrix Trichophyta*

Dr. G. F. Donald of Adelaide, South Australia contributed a paper on "The Endothrix Trichophyta; with Special Reference to the Similarity of the Clinical Disease caused by *T. tonsurans* and *T. violaceum*".* Investigation of the cases of tinea capitis among the aboriginal and part-aboriginal population of South Australia had shown the overwhelming preponderance of infections due to the endothrix trichophyta. In the period 1952 to 1960 endothrix trichophyta had caused 154 out of 155 cases of tinea capitis seen in coloured people but only 39 out of 484 cases in white people examined; 85 were due to *T. tonsurans,* 53 to *T. violaceum,* and 12 cases had double infections. Adult infections, nail and skin involvement were not common. Scarring was commonly seen in both types of infection but appeared to be more frequent in *T. violaceum* infections. The same clinical appearances were caused by both fungi and it was not possible to predict the causative pathogen from the clinical features. An inflammatory host reaction was seen more commonly among the 39 white persons studied than in the aboriginal group. Variations in the clinical disease due to *T. tonsurans* and *T. violaceum* have been recorded in the medical literature. It is believed that these variations were largely due to the relative importance of the 2 fungi in various parts of the world, and the differing racial groups infected. It is unlikely that there was a significant difference in the intrinsic pathogenicity of *T. tonsurans* and *T. violaceum.*

Brisk discussions followed the reading of these 4 papers and it was considered that this, the first Symposium on Medical Mycology to be held at an A.N.Z.A.A.S. Congress, was very successful.

E. B. DURIE

## SOCIETY NEWS

### MEETING OF ISHAM IN MONTREAL AUGUST, 1962

In addition to the symposium on "The Influence of the Environment on the Epidemiology of Mycoses" to be held at the 8th International Congress of Microbiology and at which Dr. C. W. Emmons will be chairman there will be several meetings of the Society. Arrangements have been made for a meeting of the Executive Committee on the evening of Friday August 17th and a general business meeting of ISHAM at 2 p.m. on Sunday 19th August. One Focal Topic Session for delivery of papers on Human and Animal Mycology has also been arranged.

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* These papers have been accepted for publication in *The Australian Journal of Dermatology.*

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# A NEW SPECIES OF *MICROSPORUM* PATHOGENIC TO MAN AND ANIMALS

Lucille K. Georg* L. Ajello*
Lorraine Friedman† Sherry A. Brinkman*

* *Communicable Disease Center, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta 22, Georgia.*
† *Department of Microbiology, Tulane University School of Medicine, New Orleans 12, Louisiana.*

A new dermatophyte species, *Microsporum vanbreuseghemii*, is described. This fungus was isolated from ringworm of a Malabar squirrel, a dog, and 2 human beings. The fungus is characterized by the development of many large, thick-walled, echinulate, cylindro-fusiform macroconidia. Cleistothecia with ascospores were produced by all strains when grown on hair on the surface of soil. It is proposed that the perfect form of *M. vanbreuseghemii* be included in the genus *Nannizzia* as *N. grubyia* sp. nov.

A new dermatophyte species of the genus *Microsporum* and its perfect form is described. This description is based on studies of isolates from ringworm in a Malabar squirrel, a dog, and 2 human beings.

The fungus was first isolated from a ringworm infection in a Malabar squirrel which had died at the Chicago Zoological Park, Brookfield, Illinois. This isolate, as well as infected tissues from the host, were studied at the Mycology Unit of the Communicable Disease Center in 1959 (Georg, Kaplan, Ajello, Williamson, & Tilden, 1959). At that time, the authors believed the organism to be *Keratinomyces ajelloi* (Vanbreuseghem, 1952). It differed from the description given by Vanbreuseghem, however, in that it lacked a reddish to purple pigment on the reverse of the colony, and produced numerous microconidia which had not been described by Vanbreuseghem. Also the squirrel isolate readily produced ringworm in the guinea pig, while Vanbreuseghem's strain, as well as soil strains isolated in this country by Ajello, did not produce infection in experimental animals.

Since, in a restudy of Vanbreuseghem's original isolate a few microconidia were discovered, and since many soil isolates did not produce a purple pigment, the description of *K. ajelloi* was emended to include the microconidia and the fact that pigmentation may be lacking, as well as a description of the parasitic nature of the fungus.

In November, 1960, a somewhat similar isolate—at first believed also to be *K. ajelloi*—was sent to us by Dr. A. E. Blum, Crystal City, Missouri, through the Division of Health Laboratories, Jefferson City, Missouri. This isolate had been obtained from a case of ringworm in a dog. It differed from the squirrel strain in that the surface of the colony was a deep pink color and the macroconidia were markedly echinulate.

One month later (Dec., 1960), a similar isolate, from a case of human ringworm, was obtained by one of us (L.F.). This human strain appeared to be identical to the canine isolate. A description of this strain and the infections observed in 2 humans will be described by Henington, Friedman, Perrett, & Kennedy (In press).

## OBSERVATIONS

### 1. *Description of the Canine Isolate*

The culture in question, which had been isolated from skin scrapings obtained from a dog, was at first identified as *K. ajelloi*. A request was made for additional clinical materials and a description of the lesions on the diseased dog.

A portion of the fresh skin scrapings was examined directly in 10% KOH. Branched, septate mycelium with arthrospores was present in the skin scales. The remainder of the material was inoculated into several tubes of Sabouraud glucose agar containing chloramphenicol and cycloheximide. After incubation at 25° C., pure cultures similar to the original isolate were obtained. No other dermatophyte was recovered.

This canine isolate proved to be pathogenic when inoculated into the scarified skin of 2 guinea pigs. Skin scrapings from the guinea pigs revealed the presence of branched mycelium. Hairs were invaded by the fungus, and showed a septate branched mycelium in their interiors. Masses of large arthrospores were seen on the surfaces of several of the infected hairs. These did not, however, form a distinct sheath about the hair. Fluorescence of infected hairs under a Wood's lamp was not observed.

A description of the infected dog was provided by Dr. Blum. The host was a 3-month-old female boxer, living in Kimmswick, Missouri. It had several circular, scaly lesions about 2·5 cm. diam. on 1 shoulder and flank. The patches showed loss of hair. The dog has been treated with topical applications of nystatin and its lesions cleared in a week. Photographs could not be obtained.

On Sabouraud dextrose agar, the canine isolate grew rapidly and produced a flat colony. Its surface was coarsely granular and pink to deep rose in color. The reverse of the colony was yellowish. Numerous multiseptate macroconidia were produced. These were cylindro-fusiform in shape and had thick walls. The thickness of the walls, 2-2·5μ diam. in mature spores, was striking. The average number of cells in the macroconidia was 6-7. However, many extremely long spores with 12-13 cells were observed. These ranged from 43·8-87·5μ long by 8·0-13·5μ wide (Average: 61·7μ × 10·6μ). The macroconidia resembled those described for *K. ajelloi* by Vanbreuseghem. However it was noted that they were slightly longer and the surface of the spores was markedly echinulate.

Microconidia were also produced and these were fairly numerous in some areas of the culture. They were single-celled, pyriform to obovate, and borne along the sides of the aerial hyphae.

Attempts were made to induce sexual reproduction. This was done by growing the fungus on moist, sterile soil, baited with 1 cm. long bits of sterilized horse hair according to the method described by Dawson & Gentles (1961) for the production of cleistothecia in *K. ajelloi*.

In the first attempt, the soil and horse hair was sterilized by ethylene oxide gas; in the second and third attempts the soil was autoclaved and baited with either autoclaved horse hair or autoclaved human hair. In all trials, cleistothecia were produced in abundance. In some instances they appeared as early as 5 days after inoculation. Upon microscopic examination of these structures it was recognized that the cleistothecia produced were not those of *Arthroderma uncinatum,* the perfect form of *K. ajelloi* (Dawson & Gentles, 1959, 1961), but were similar to those of the genus *Nannizzia* recently described by Stockdale (1961).

As a result of these findings it was recognized that the canine isolate was not *K. ajelloi.* The fact that the macroconidia were echinulate suggested that the organism should be placed in the genus *Microsporum* and this was further substantiated by the fact that the cleistothecia were characteristic of the genus *Nannizzia.* This genus was established in 1961 by Stockdale to accomodate the sexual stage of *M. gypseum* which she named *N. incurvata.* The perfect stage of *M. nanum* described by Dawson & Gentles (1961) and the perfect stage of *M. cookei* described by Ajello (1961) also fall in this same genus.

The fungus isolated from the dog was named *Microsporum vanbreuseghemii* in honor of the distinguished Belgian mycologist, Dr. Raymond Vanbreuseghem.

**Microsporum vanbreuseghemii** sp. nov.

*Colonia* plana, superficies pulverea vel lanuginosa, in colorem albam ad luteam vel rubicundam. Pars aversa coloniae sine colore vel luteam. Mycelium ramosum; hyphis hyalinis, septatis 2-5μ D.

*Macroconidia* portaverunt in abundantiam singillatim, lateratim vel terminatim cylindrofusiforma, multiseptata, cum parietibus crassis (2-2·5μ crassis) et asperulatae, densae generatim 7-10 septatis. Dimensiones 58·8-61·7μ × 10·4-10·6μ.

*Microconidia* portaverunt lateratim in abundantiam aut paucitatiam, piriforma ad abovata. Dimensiones circiter 9·2 × 4·4μ. Degeneratio pleomorphicum celeriter evolvit. Parasitus de bestiae et hominis est. Cornuaffinitiva.

*Typus*: ATCC 14419.

*Colony* flat, surface powdery or downy, cream to yellow or pink in color. Reverse of colony colorless or yellow. Mycelium branched; hyphae hyaline, septate 2-5μ D.

*Macroconidia* borne in abundance singly, laterally or terminally; cylindrofusiform, multiseptate, thick-walled (2-2·5μ thick) and densely echinulate, generally 7-10 septations. Dimensions 58·8-61·7μ × 10·4-10·6μ.

Microconidia borne laterally abundantly or sparsely, pyriform to obovate. Dimensions around 9·2 × 4·4μ. Pleomorphic degeneration develops early. Parasite of animals and man. Keratinophilic.

*Typus*: ATCC 14419.

### II. *Comparison of the Canine Isolate to the Isolates from the Malabar Squirrel and the Human Infection*

A restudy of the Malabar squirrel isolate and 1 of the human isolates revealed that these were essentially similar to the canine strain. Both the dog and human strains produced flat, powdery colonies with pink to deep rose surface pigment and a pale yellow pigment on their reverse sides. When first studied, the squirrel strain had presented a white to cream colored surface with little or no pigment on its reverse side. However, after having been in stock culture for over six years, it was recultured, and in several transfers, areas of pink pigmentation developed. The macroconidia of the 3 strains were similar with the exception that echinulations did not occur as regularly on the macroconidia of the squirrel isolate. However, following growth on soil and hair plates, the macroconidia of this strain became markedly echinulate. Measurements of the microconidia and macroconidia of these 3 strains as well as measurements of the spores of 5 strains of *K. ajelloi* are given in table 1.

| *Structure* | *Organism* | | *Number Measured* | *Range* | *Average* |
|---|---|---|---|---|---|
| MACROCONIDIA<br>Length | *M. vanbreuseghemii* | X-127* | 100 | 44·4-72·0μ | 58·8μ |
| | | X-321 | 400 | 34·8-78·0μ | 59·0μ |
| | | X-322 | 200 | 43·8-87·5μ | 61·7μ |
| | *K. ajelloi* | X-125 | 200 | 30·0-60·0μ | 44·7μ |
| | | X-126 | 100 | 24·0-64·8μ | 46·9μ |
| | | X-132 | 100 | 24·0-68·4μ | 51·5μ |
| | | X-133 | 100 | 30·0-80·4μ | 54·2μ |
| | | X-134 | 100 | 24·0-61·2μ | 42·5μ |
| Width (widest point) | *M. vanbreuseghemii* | X-321 | 50 | 9·0-11·5μ | 10·4μ |
| | | X-322 | 50 | 8·0-13·5μ | 10·6μ |
| | *K. ajelloi* | X-125 | 50 | 7·7-13·9μ | 10·1μ |
| | | X-133 | 50 | 4·8-12·0μ | 8·5μ |
| MICROCONIDIA<br>Length | *M. vanbreuseghemii* | X-127 | 20 | 6·0-13·2μ | 9·2μ |
| | *K. ajelloi* | X-134 | 20 | 6·0-13·2μ | 9·2μ |
| Width (widest point) | *M. vanbreuseghemii* | X-127 | 20 | 3·6-6·0μ | 4·0μ |
| | *K. ajelloi* | X-134 | 10 | 3·6-7·2μ | 4·7μ |

* Source of Strains: X-125 Belgium soil (Vanbreuseghem), X-127 Malabar squirrel (Georg *et al.*), X-132 New Jersey soil (Georg), X-133 West Virginia soil (Ajello), X-134 Alaska soil (Ajello), X-321 Human (Henington *et al.*), X-322 Dog (Georg *et al.*).

TABLE 1.—SPORE MEASUREMENTS OF *Microsporum vanbreuseghemii* AND *Keratinomyces ajelloi*

All 3 isolates readily produced infection when rubbed onto the shaved and slightly scarified skin of guinea pigs. The skin and hairs of the experimental animals were invaded by fungus elements. True ectothrix sheaths of fungus spores were not observed in these animals, and hairs did not fluoresce under a Wood's lamp. It should be noted however, that fluorescent hairs were observed in the human infections observed by Henington *et al* (In press).

All 3 isolates were cultured on autoclaved soil with autoclaved hair (either human or horse hair) and all 3 produced similar cleistothecia that were referrable to the genus *Nannizzia*.

It was concluded that the 3 strains represented the same species, *Microsporum vanbreuseghemii.*

### III. *Attempts to Produce Ascospores in M. vanbreuseghemii*

Although all 3 strains produced cleistothecia on soil and hair plates, the cleistothecia remained sterile after 6 weeks' incubation.

Attempts were made to produce fertile cleistothecia by cross-mating the strains. All possible combinations were made. It was found after 2 weeks that the combination of the human and canine isolates as well as the combination of the human and squirrel strains produced ascospores.

However, 1 week later the soil plates which had been inoculated separately with the original isolates were re-examined. At this time (after 9 weeks), 1 of the human strains was producing mature cleistothecia with ascospores. Single ascospore cultures were isolated from mature cleistothecia by Miss Christine Dawson, Department of Bacteriology, University of Glasgow. Through the mating of these isolates, she obtained evidence that *M. vanbreuseghemii* is heterothallic.

Mature cleistothecia with ascospores could be spotted on the plates because they developed a buff color following the production of numerous yellowish ascospores. Rupture of the cleistothecia under a coverslip allowed the escape of the asci, which contained 8 pale yellow ascospores.

Cleistothecia at first were white, but they became buff to tan when mature. They were spherical and irregularly covered with macroconidia and a few spirals. They ranged from 150-600μ in diameter, exclusive of appendages. The peridium consisted of interwoven, branched, septate hyphae ranging from 3-7·5μ in width. The cells of the peridium were thick-walled, densely echinulate with a slight gradual constriction at the central area of most cells so that these cells were slightly broader near the septations. In general shape, they resembled the phalangeal bones of the human hand. The peridial hyphae branched both dichotomously and uncinately. Terminal branches most commonly curved away from their main axis. Many of the terminal cells tapered to a rounded point or tapered into a short coil of 1-3 turns (2-2·5μ diam.) that was curled under the arc of the decurved hyphal branch from which it had arisen. In some instances, these appendages were extremely long and delicate. They tapered to a uniform diameter of 1-1·5μ and were tightly coiled with 30-50 turns. These delicate spirals were usually contained within the meshes of the peridium, however with slight pressure of the coverslip, they partially uncoiled and extended several hundred microns from the edge of the cleistothecium. The coils were extremely numerous on the young cleistothecia, but they disappeared when the cleistothecia matured. Other peridial hyphae had blunt ends or bore stump-like protuberances along their sides. These blunt structures represented the conidiophores of the macroconidia. Such spores were borne in great numbers, both terminally and laterally on the peridial hyphae. The macroconidia are detached so easily that they seldom remain attached to the peridial hyphae.

When the cleistothecium was crushed, a large number of asci escaped. These asci were spherical or nearly so with an average diameter of 6μ. The ascus wall was evanescent and 8 ascospores were easily released. These were pale yellow, oval, and averaged 3 by 2·4μ. They were smooth-walled and devoid of ornaments.

This perfect fungus was named *Nannizzia grubyia* in honor of the Hungarian physician, Dr. David Gruby, who first described the genus *Microsporum* in 1843.

**Nannizzia grubyia** sp. nov.

Fungus heterothallicus cleistothecia globosa, alba ad pallido-bubalina, 150-600μ D. appendiculis excludent. *Hyphae peridii* hyalinae, septatae, ramosae bifurcae et uncinativae, plerumque curvescent ad latus ab hypha principe remotius. Cellae modice crasso-parietales, asperulatae densae aequaliter modice constrictae ad centram (similis ad ossibus digitorum manus) degeratim 4-5μ D. interdum 7·5-10·0μ. Hyphae peridae cum apices liberes terminant retusatim vel sicut punctum rotundum, vel tenuant gradatim in hyphae glomerant solute 2-3 flexibus, vel in hyphae elongate (usque ad 300μ longae), parietibus tenuibus laevibus, hyphae septatae tenuant gradatim ab 3·0 ad 1·5μ D. ab usque 30-50 glomeri stricti. *Macroconidia* crassi parietis cylindrofusiforma, multiseptata et asperulatae densae portaverunt lateratim et terminatim super hypha peridii. *Asci* globosi, tenibus parietibus, evandi 4·8-6·0μ D. octospori. *Ascospori* hyalini, pallido-luteam cum parietibus laevibus, ovati 2·4 × 3·0μ.
*Stat. conid.*: *Microsporum vanbreuseghemii*
*Habitat*: Cutis et pilus de bestiae et hominis.
*Typus*: ATCC. 14422(+), ATCC. 14423(−)

Heterothallic. Cleistothecia globose, white to pale buff 150-600μ D. exclusive of appendages. *Peridial hyphae* hyaline, septate, branching dichotomously and uncinately, mostly curved to one side, the outside of the main hypha. Cells moderately thick-walled, densely echinulate, moderately constricted at their central part (similar to phalanges of hand) generally 4-5μ D; occasionally 7·5-10·0μ. Free ends of peridial hyphae terminate bluntly or as rounded points, or taper into smooth-walled loosely coiled hyphae of 2-3 turns, or into elongate (up to 300μ long) thin, smooth-walled, septate hyphae tapering from 3·0-1·5μ D. with up to 30-50 tight coils. *Macroconidia* thick-walled, cylindro-fusiform, multiseptate and densely echinulate, borne laterally and terminally on peridial phyhae. *Asci* globose, thin-walled, evanescent 4·8-6·0μ D. eight-spored. *Ascospores* hyaline, pale yellow, smooth-walled, ovate 2·4 × 3·0μ.
*Stat. conid.*: *Microsporum vanbreuseghemii*
*Habitat*: Skin and hair of animals and man.
*Typus*: ATCC. 14422(+), ATCC. 14423(−)

### IV. *Comparison of the Imperfect and Perfect Forms of M. vanbreuseghemii and K. ajelloi*

Because of the superficial similarity of these 2 species, the *M. vanbreuseghemii* strains were compared with several soil isolates of *K. ajelloi*, as well as those strains of this organism which had been isolated from ringworm in the horse by Pier in the United States (1961) and by Rieth & El-Fiki in Germany (1959).

All of the *K. ajelloi* strains produced flat, powdery to slightly fluffy colonies. Their surface pigment ranged from light cream to orange-tan. On the reverse of the colonies, a reddish-purple pigment was produced by some strains. Several of the soil isolates, however, had no pigment on reverse. Microconidia were present in all, although they were rare in several strains. Macroconidia were

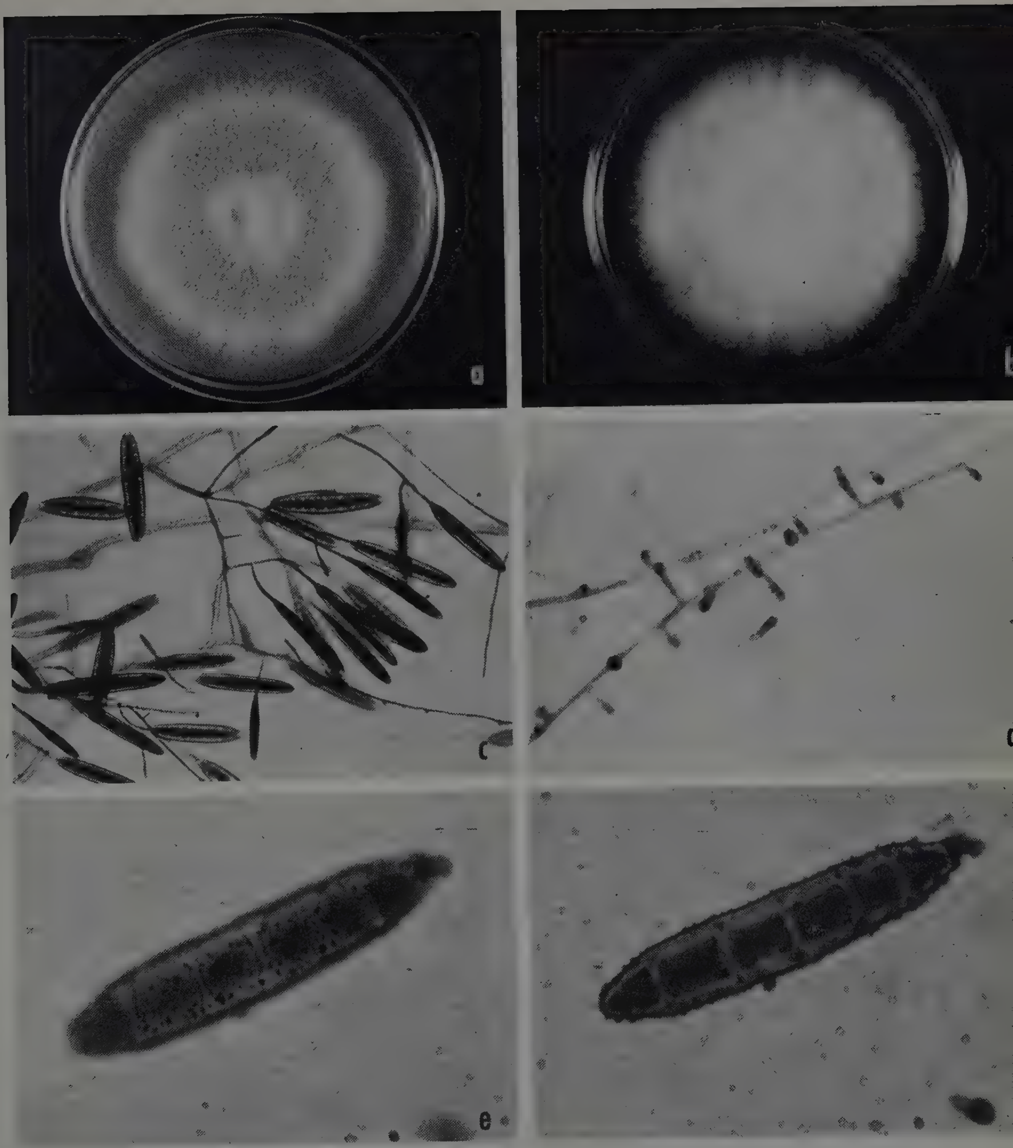

PLATE I—*Microsporum vanbreuseghemii*

a. Powdery colony (strain X322). Sabouraud dextrose agar, 4 weeks.
b. Fluffy colony (strain X127). Sabouraud dextrose agar, 4 weeks.
c. Macroconidia (strain X321). Slide culture, showing manner by which spores are borne on mycelium, 180 ×.
d. Microconidia (strain X127), 900 ×.
e. Macroconidium (strain X322), showing echinulate surface, 300 ×.
f. Macroconidium (strain X322). Median optical view showing echinulations on edge of spore, 300 ×.

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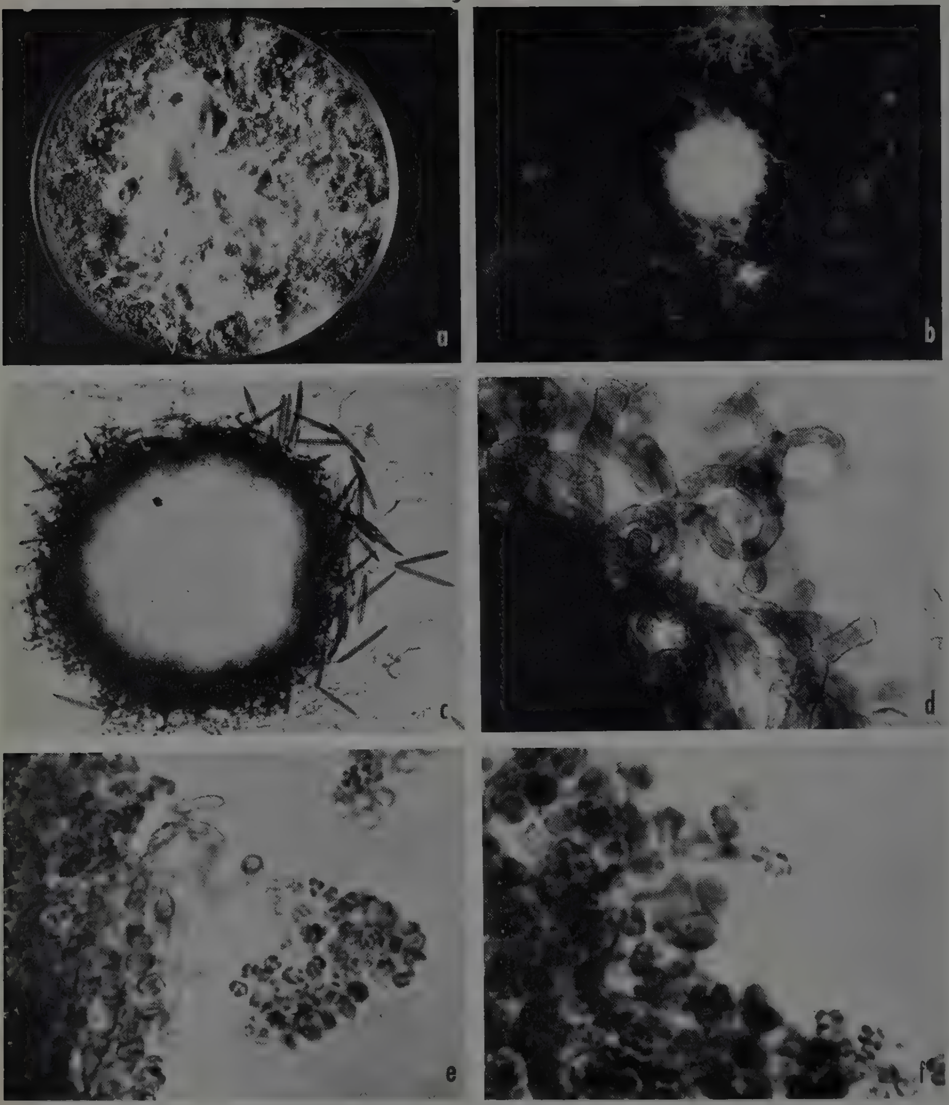

PLATE II—*Nannizzia grubyia*

a. Soil plate with horse hair showing cleistothecia.
b. Single cleistothecium from surface of soil plate, 30 ×.
c. Mature cleistothecium (strain X438), 54 ×.
d. Edge of mature cleistothecium (strain X438), 400 ×.
e. Asci and ascospores (strain X438), 400 ×.
f. Asci and ascospores (strain X438), 670 ×.

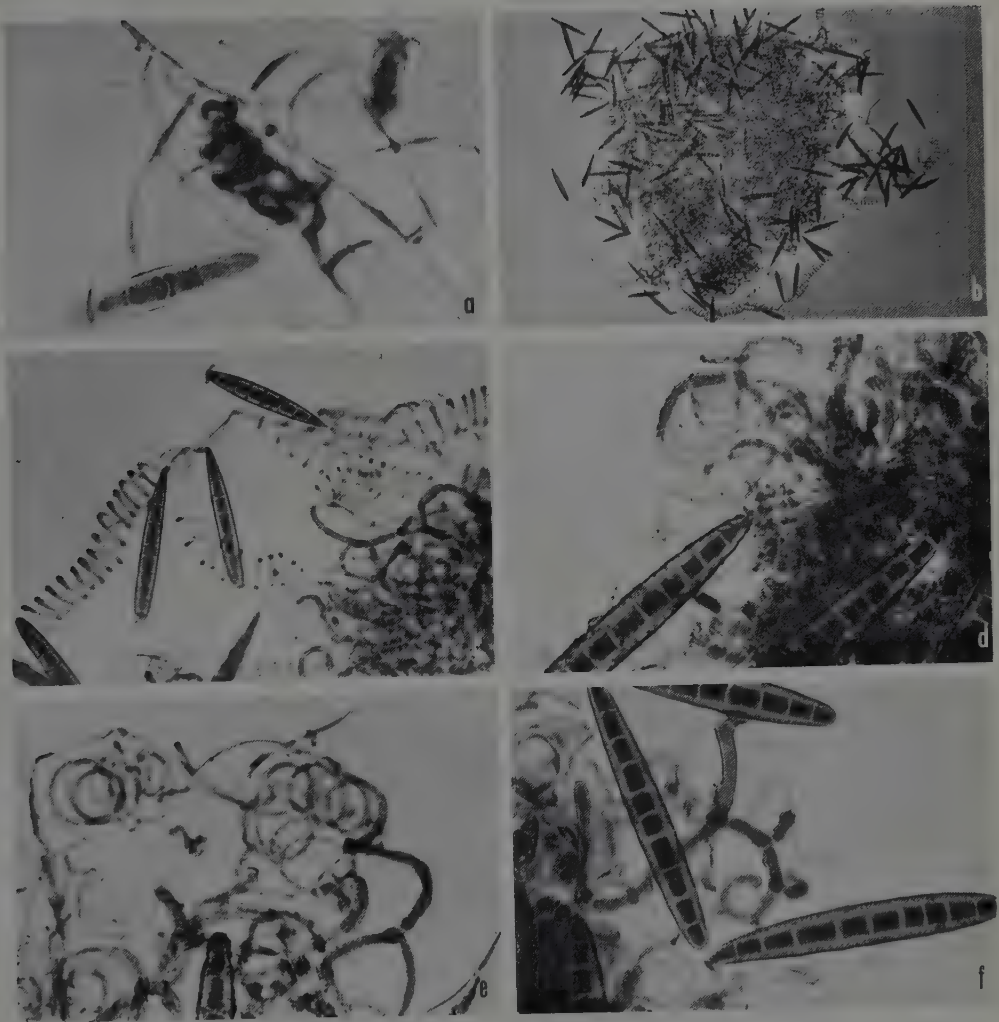

PLATE III—*Nannizzia grubyia*

a. Cleistothecial initial (strain X322), 400 ×.
b. Immature cleistothecium (strain X322), 12 ×.
c. Detail of immature cleistothecium showing spiral appendages (strain X322), 54 ×.
d. Peridial hypha with tightly coiled spiral appendage (strain X321), 400 ×.
e. Peridial hypha with partially uncoiled spirals (strain X321), 400 ×.
f. Peridial hyphae showing macroconidial attachment scars (strain X127), 400 ×.

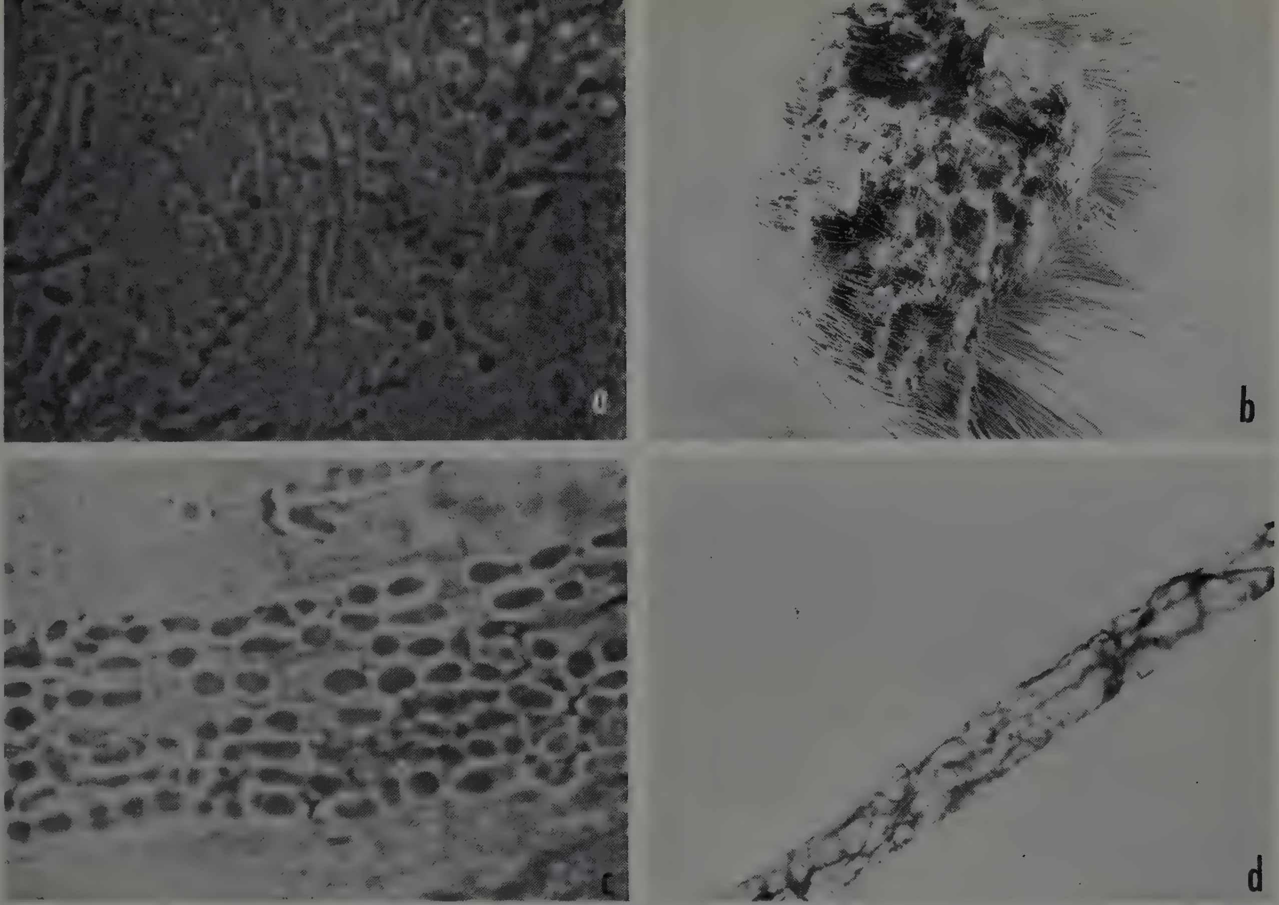

PLATE IV—Skin and hair infections due to *Microsporum vanbreuseghemii*

a. Skin scraping from natural infection in dog showing mycelium and arthrospores, 300 ×.
b. Experimental lesion in guinea pig.
c. Guinea pig hair showing large spores on surface of hair, KOH mount, 300 ×.
d. Guinea pig hair showing mycelium in interior of hair, PAS stain, 300

numerous in all strains. They were essentially similar in size and shape to those of *M. vanbreuseghemii*, however, none of them were echinulate. Measurements of the macroconidia revealed that those of *M. vanbreuseghemii* were slightly longer (table I). Attempts to produce cleistothecia were successful with both Pier's and Rieth & El-Fiki's isolates. The cleistothecia were characteristic of *Arthroderma uncinatum*, the perfect form of *K. ajelloi* first described by Dawson & Gentles (1961).

Table 2 compares the significant characteristics of the imperfect and perfect forms of *K. ajelloi* and *M. vanbreuseghemii*.

| *Diagnostic Characteristics* | *Keratinomyces ajelloi* | *Microsporum vanbreuseghemii* |
|---|---|---|
| Gross colony characteristics | Colony rapid growing, flat with powdery to fluffy surface. Surface color white to cream, to orange-tan. Pigment on reverse reddish, becoming deep purple, or no pigment. | Colony rapid growing, flat with powdery to fluffy surface. Surface color white to yellowish or pink to deep rose. May become tan. Pigment on reverse light yellow to bright lemon-yellow or lacking. |
| Macroconidia | Macroconidia numerous, cylindro-fusiform with thick, smooth walls, multiseptate with 5 to 12 sections.<br>Size: 42·5-54·2μ × 8·5-10·1μ | Macroconidia numerous, cylindro-fusiform with thick, echinulated walls, multiseptate with 5 to 12 sections.<br>Size: 58·8-61·7μ × 10·4-10·6μ |
| Microconidia | Present in some strains, pyriform to obovate.<br>Size: 9·2 × 4·7μ | Usually numerous, pyriform to obovate.<br>Size: 9·2 × 4·0μ |
| Sexual reproductive forms | *Arthroderma uncinatum* (Dawson & Gentles, 1961) Heterothallic. *Cleistothecia* globose, white to buff 300-900μ D. *Peridial hyphae* hyaline, septate, uncinately branched usually away from main hypha. Cells thick-walled, densely echinulate, dumb-bell shaped 4-7μ D. *Spirals,* smooth-walled, septate of narrow diameter, borne laterally or terminally on peridial hyphae, vary in length and number of turns. *Macroconidia* smooth, thick-walled, septate, cylindro-fusiform borne laterally and terminally on peridial hyphae. *Asci* globose, thin-walled, evanescent 4·9-7·2μ D. 8-spored. Ascospores hyaline, smooth or finely roughened, lenticular 1·6 × 2·5μ, light yellow. | *Nannizzia grubyia*<br>Heterothallic. Cleistothecia globose white to buff 150-600μ D. *Peridial hyphae* hyaline, septate, uncinately branched usually away from main hypha. Cells thick-walled, densely echinulate, phalangiform 2-7·5μ D. *Spirals,* smooth walled, septate, of narrow diameter, borne terminally on peridial hyphae, vary in length and number of turns. *Macroconidia* densely echinulate, thick-walled, septate, cylindro-fusiform borne laterally or terminally on peridial hyphae. *Asci* globose, thin-walled, evanescent 4·8-6·0μ D. 8-spored. Ascospores hyaline, smooth, oval 2·4 × 3·0μ light yellow. |
| Pathogenic properties | This species has been described as a cause of spontaneous ringworm in horses (Reith and El Fiki, 1959; Pier, 1961). Experimental infections in mice, guinea pigs, and a human have been obtained with a soil isolate by Evolceanu & Alteras (1959). | This species has been described as the cause of spontaneous ringworm in a Malabar squirrel, a dog, and two humans. Experimental infections readily produced in the guinea pig. |
| Source in nature | Common in soil in many parts of the world (Ajello, 1960). | Not known. |

Table 2.—Comparison of Imperfect and Perfect Forms of *K. Ajelloi* and *M. Vanbreuseghemii*

## STRAINS AVAILABLE

Three strains of *M. vanbreuseghemii*, and single ascospore cultures of *N. grubyia* have been deposited with the American Type Culture Collection. The culture collection numbers are listed below.

| | |
|---|---|
| ATCC—14419— | *Microsporum vanbreuseghemii* (type culture). Isolated at Mycology Unit, Communicable Disease Center, from ringworm in a dog, Crystal City, Mo. |
| ATCC—14420— | *M. vanbreuseghemii*. Isolated at Laboratory of Mycology, Tulane University School of Medicine, from ringworm of the scalp in a young girl. |
| ATCC—14421— | *M. vanbreuseghemii*. Isolated at Mycology Unit, Communicable Disease Center, from ringworm in a Malabar squirrel, Chicago Zoological Park, Brookfield, Illinois. |
| ATCC—14422(+) | *Nannizzia grubyia*. Single ascospore culture prepared from cleistothecia formed by mating of strains X322 & X321. |
| ATCC—14423(−) | *Nannizzia grubyia*. Single ascospore culture prepared from cleistothecia formed by mating of strains X322 & X321. |

## RÉSUMÉ

Une nouvelle espèce de dermatophyte, *Microsporum vanbreuseghemii* est décrite. Ce champignon a été isolé de teignes tondantes humaines (2 cas) et animales (un écureuil de Malabar et un chien). Le champignon est caractérisé par le développement de plusieurs grandes macroconidies, cylindrofusiformes, échinulées, à parois épaisses et dont les dimensions sont 58·8-61·7μ × 10·4-10·6μ. Des cleistothèces avec ascospores ont été produites par trois souches, après culture sur des crins de cheval disposés sur de la terre en boite de Petri. La forme parfaite de *M. vanbreuseghemii* appartient au genre *Nannizzia* et reçoit le nom de *N. grubyia* sp. nov.

## ACKNOWLEDGEMENTS

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# TRICHOPHYTIN REACTION IN NATURAL AND EXPERIMENTAL DERMATOPHYTOSIS AND IN TUBERCULOSIS*

H. Neves
with the technical assistance of J. S. Custódio and M. Manuela Figueiredo

*Mycology Laboratory, Department of Dermatology, Faculty of Medicine, University of Lisbon, Portugal (Chairman: Prof. J. Esteves)*

In a comparative study of the trichophytin reaction in 170 cases of ringworm and 571 subjects without apparent infection it was found that:

1. Immediate reaction of urticarial type was very rare and non infiltrative erythema was not related to the presence of dermatophytes, occurring equally in both groups and independently of the result of delayed response.
2. Delayed reactions could only be considered significant when strongly positive (infiltrative erythema with papules and vesicles, larger than 30 mm. in diameter), which appeared almost exclusively in cases of deep inflammatory ringworm.
3. The presence of active tuberculosis or tuberculin sensitivity did not influence the response to trichophytin.
4. Experimental inoculation with dermatophytes only modified trichophytin sensitivity when inflammatory lesions were produced.

Reports concerning the relationship of a positive trichophytin reaction to the presence or absence of dermatophytosis have varied considerably. This has been attributed to constitutional factors, to the lack of a uniform test material, to the species of infecting organism and to whether the lesions are chronic or acutely inflammatory (Götz, 1959).

The present study describes the results of the trichophytin test in persons with and without dermatophytosis. In addition the effects of tuberculosis and of human experimental dermatophytosis on the trichophytin reaction were studied.

## Material and Methods

Trichophytin tests were performed on 170 patients with ringworm (103 of the scalp, 19 of the beard, 7 on glabrous skin, 6 in the groins, 6 of the feet, 6 of the nails and 23 with multiple sites) and on 571 persons without any clinically apparent dermatophyte lesion. The latter included 242 patients with pulmonary tuberculosis, 287 with non-mycotic dermatoses and 42 with miscellaneous systemic diseases.

The preparation used was "Trichophytin Hoechst"†. According to the manufacturer this is prepared from cultures of *Trichophyton gypseum* (*T. mentagrophytes*), *T. cerebriforme* (*T. tonsurans*), *T. rosaceum* (*T. megnini*), *T. faviforme* (*T. verrucosum*) and *T. violaceum*. The 6 weeks old cultures in meat-extract-peptone broth are autoclaved and filtered. The extract is then concentrated at 80-90° C. to one seventh of its original volume and preserved in 0·5% phenol. Each lot of trichophytin is tested for sterility and injected subcutaneously in normal guinea pigs and mice to rule out non-specific reactions.

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* Work done under a grant from Instituto de Alta Cultura, Portuguese Ministry of Education.

† Farbwerk Hoechst A G, Frankfurt M., Germany.

Preliminary tests showed that a 10% solution of this material produced intense reaction in the majority of patients with ringworm and of uninfected controls, while 5% and 2% solutions produced uniform responses, positive in cases of kerion and negative in most patients with non-inflammatory ringworm and in normal controls. Each person therefore was injected intradermally with 0·1 ml. of a 2% solution and observed after 15 minutes and 48 hours.

Positive immediate reactions were recorded if erythema and infiltration of the urticarial type occurred within 15 minutes. The reaction was regarded as negative if there was no alteration at the injection site or if non-infiltrative erythema only occurred.

Delayed reactions were grouped in 3 degrees of severity, according to Götz (1959): 1+ an inflammatory erythema of 10-20 mm. diam. with little infiltration; 2+ an inflammatory erythema of 20-30 mm. with infiltration or papule formation; 3+ an inflammatory erythema larger than 30 mm. with infiltration, papules and vesicles formation.

## RESULTS

*Immediate reactions.* None of the 170 ringworm patients had positive immediate reactions and only 1 of the 466 uninfected subjects observed was positive. A slight non-infiltrative erythema (negative by the adopted criteria) followed the injection in 5% of patients with dermatophytoses and in 10% of the uninfected controls.

*Delayed reactions.* Table 1 compares the incidence of delayed reactions in infected and uninfected subjects. It will be seen that positive reactions developed in 41% of the patients with ringworm and in 23% of uninfected controls. Whereas 22 cases with dermatophytoses gave 3+ reactions, only 3 without infection behaved this way. Two of these denied any previous infection, the third referred to a prior infection of the beard which might have been kerion barbae. One patient with psoriasis gave a non-specific response in the form of the Koebner phenomenon; another with leukemia gave a purpuric reaction which he tended to do to all injections.

| *Clinical condition* | *No. of patients* | *No. positive* | *Grade of reactions* | | |
|---|---|---|---|---|---|
| | | | 1+ | 2+ | 3+ |
| Dermatophytoses | 170 | 69 (41%) | 19 (11%) | 28 (17%) | 22 (13%) |
| Uninfected | 571 | 130 (23%) | 72 (13%) | 55 (10%) | 3 (0·5%) |

TABLE 1.—INCIDENCE AND SEVERITY OF DELAYED REACTIONS IN INFECTED AND UNINFECTED PATIENTS

Table 2 compares the incidence of positive reactors in patients with and

| *Clinical condition* | *No. of patients* | *No. positive* | *Grade of reaction* | | |
|---|---|---|---|---|---|
| | | | 1+ | 2+ | 3+ |
| Inflammatory | 35 | 34 | 1 | 12 | 21 |
| Non-inflammatory | 135 | 35 | 18 | 16 | 1 |
| Total | 170 | 69 | 19 | 28 | 22 |

TABLE 2.—INCIDENCE AND SEVERITY OF DELAYED REACTIONS IN PATIENTS WITH INFLAMMATORY AND NON-INFLAMMATORY RINGWORM

without inflammatory lesions. The distinction of both groups was based on clinical features (presence or absence of marked erythema, oedema, vesicles and suppuration) and evolution (usually longer in non-inflammatory forms). It will be seen that in those with inflammatory ringworm there was a considerably higher incidence of positive reactors (97 % compared with 26 %) and that the reactions in this group were more severe, most of them being of a 3+ rating. In the non-inflammatory ringworms only 1 patient gave a severe reaction. This was a 6 year old girl with tinea capitis caused by *T. violaceum* and a concomitant ascariasis.

As shown in table 3 the most frequent infecting organisms were *T. violaceum*, *T. tonsurans*, *T. megnini* and *Microsporum canis*. The numbers of the other species were too small to warrant any conclusions but it is clear that the species of the infecting organism did not affect the development of hypersensitivity. Even patients with *M. canis* infections reacted in about the same proportion as the others although *M. canis* was not among the organisms included in the preparation of the trichophytin. It can be seen however that none of the *M. canis* infections was inflammatory and that infections by *T. violaceum* were less frequently inflammatory than the others. Many (58 %) of the patients were children with tinea capitis. No significant relationships were observed between the occurrence of positive reactions and the site of infection, age or sex of the cases.

| *Organism* | *No. of patients* | *No. with inflammatory lesions* | *No. positive* | *Grade of reaction* | | |
|---|---|---|---|---|---|---|
| | | | | 1+ | 2+ | 3+ |
| *T. violaceum* | 51 | 3 | 18 | 5 | 10 | 3 |
| *T. tonsurans* | 27 | 7 | 11 | 5 | 1 | 5 |
| *T. megnini* | 19 | 4 | 9 | 2 | 5 | 2 |
| *T. mentagrophytes* | 13 | 7 | 11 | 2 | 3 | 6 |
| *T. rubrum* | 7 | 1 | 1 | 0 | 0 | 1 |
| *M. canis* | 25 | 0 | 4 | 3 | 1 | 0 |
| Others or mixed | 28 | 13 | 15 | 2 | 8 | 5 |
| Total | 170 | 35 | 69 | 19 | 28 | 22 |

TABLE 3.—RELATIONSHIP BETWEEN INFECTING ORGANISMS AND DELAYED REACTIONS

*Delayed reactions in tuberculosis.* The presence of pulmonary tuberculosis did not affect the trichophytin response in patients without mycotic infection. Table 4 shows that the incidence and severity of the reaction in these 2 groups were very similar.

| *Clinical condition* | *No. of patients* | *No. positive* | *Grade of reaction* | | |
|---|---|---|---|---|---|
| | | | 1+ | 2+ | 3+ |
| Pulmonary T.B. | 242 | 49 (20%) | 26 (11%) | 22 (9%) | 1 (0·5%) |
| Other diseases | 329 | 81 (25%) | 46 (14%) | 33 (10%) | 2 (0·6%) |
| Total | 571 | 130 | 72 | 55 | 3 |

TABLE 4.—COMPARISON OF THE INCIDENCE OF DELAYED REACTIONS IN PATIENTS WITH TUBERCULOSIS AND WITH OTHER NON-MYCOTIC DISEASES

A comparative study of the trichophytin and the tuberculin tests was carried out in 16 children with tinea capitis. All the children had a negative immediate reaction and only 2 had a positive delayed reaction (2+) to trichophytin. The tuberculin test was positive to the dilution of 1/5000 in 4 children and to 1/1000 in 1 more. Only 1 of these patients had positive trichophytin reaction. The other positive to trichophytin was tuberculin negative. No relationship could thus be demonstrated between the trichophytin and tuberculin tests.

*Delayed reaction in experimental dermatophytosis.* In a previous report (Esteves & Neves, 1959a) *T. violaceum* was inoculated on the glabrous skin of 9 children who had had tinea capitis and 9 who had not had ringworm. Non-inflammatory lesions developed in 5 of the first group and in 4 of the latter. Trichophytin tests were performed immediately before and 7 weeks after the inoculation. In all subjects the immediate response was negative. In the group without previous ringworm the delayed response to trichophytin was the same before and after inoculation (negative in 7 and strongly positive in 2). In the group with previous tinea the reaction was not modified in 6 (negative in 4 and positive in 2). In 1 case the response was attenuated from 3+ to 1+ after successful inoculation; in another case where inoculation did not "take" the response changed from positive 3+ to negative; only 1 child, who developed an experimental lesion, changed from negative to positive (3+).

In another experiment (Esteves & Neves, 1959b), 7 adult volunteers without previous ringworm developed very inflammatory lesions after inoculation with a granular strain of *T. mentagrophytes* isolated from a kerion barbae of animal origin. The trichophytin delayed response was negative before inoculation and became strongly positive 4 weeks later in all the cases. In 2 patients the persistence of such sensitivity could be demonstrated 1 year after the experimental lesions had cleared.

## Discussion

Tests with a commercial trichophytin demonstrated a very low incidence of immediate reactions in all subjects. Slight immediate erythema was observed in 5% of 170 patients with ringworm and in 10% of non-infected controls. An urticarial immediate response was only observed in 1 case out of 636 and this had no relation with dermatophytosis. These data suggest that the immediate reaction to trichophytin is not of diagnostic significance. It has been claimed (Sulzberger, 1940; Calnan, 1958; Lewis, Hopper, Wilson & Plunkett, 1958) that an immediate reaction is usual in chronic infections by *T. rubrum,* but the reactions in 7 of such cases observed in this study did not differ from those with other dermatophytes.

The delayed reaction was negative in the majority of uninfected controls and non-inflammatory ringworm infections and was independent of the site, duration of lesion or causative agent. Strongly positive delayed reactions, with infiltrative erythema, papules, vesicles and a diameter larger than 30 mm., were observed in 21 out of 35 inflammatory cases (60%) and only in 1 of 135 non-inflammatory infections (0·7%). This agrees with previous suggestions (Muskablit & Director, 1933; Jillson & Huppert, 1949; Jung, 1953; Lindmaryr & Luger, 1954) that strongly positive reactions are generally associated with deep inflammatory lesions. The incidence of slight delayed reactions, with non-infiltrative erythema and diameter less than 20 mm., was similar in cases of ringworm (11·2 %) and uninfected controls (12·6 %); for this reason no diagnostic value can be attributed to such type of response. Delayed reactions of intermediate degree, with infiltrative erythema, papules and diameter between 20 and 30 mm. were more frequent in ringworm patients (16·5 %) than in controls (9·6 %) and even more frequent in inflammatory ringworm (34·4 %). It would appear to have some diagnostic value, but the occurrence of such reaction in uninfected controls is contradictory and cannot be explained. Perhaps the present attempts to obtain a purified antigen (Cruickshank, Trotter & Wood, 1960; Barker & Trotter, 1960; Barker, Cruickshank, Morris & Trotter, 1961) will provide better information about the real specificity of trichophytin.

There are different opinions about trichophytin reactions in tuberculosis and reciprocal sensitivity between trichophytin and tuberculin. Neisser (1903) did not find any cross-sensitivity and reported negative trichophytin reactions in lupus vulgaris. Sloimovici & Ullmo (1927) also failed to show any cross-sensitivity. However, Falk (1948) found a higher incidence of positive trichophytin reactions in subjects with positive tuberculin tests and obtained 90 % positive trichophytin reactions in cutaneous tuberculosis. In the present study the trichophytin test was negative in 193 cases (79·7 %) and slightly positive in 26 (9·1 %) of 242 patients with chronic pulmonary tuberculosis. The comparison of trichophytin and tuberculin reactions in 16 children also failed to demonstrate any relationship.

The trichophytin test was not modified after experimental inoculation of *T. violaceum* in 18 children. The reaction became strongly positive after inoculation with *T. mentagrophytes* of animal origin in 7 volunteers and the sensitivity was still demonstrable after 1 year, at least in 2 cases. Such a phenomenon was related with the highly inflammatory degree of experimental lesions produced by the latter organism. As these lesions were always self limited and spontaneously cured, one could regard their use as a vaccination if it was proved that strong sensitivity to trichophytin would mean immunity against dermatophytes. The present data differ from those reported by Rogachefsky, Rosenthal, Litt & Baer (1956), who found that exposure to dermatophytes modified previous negative trichophytin test in 52·6 % of their volunteers, although experimental lesions did not "take". These authors also reported modification of trichophytin response in 20 % of a control group not exposed to dermatophytes. However, it must be stressed that at present there is insufficient information available on the results of repeated trichophytin tests in the same individual.

## Resumo

O estudo da reacção à tricofitina em 170 casos de tinha e em 571 testemunhas sem micose aparente revelou:

1. Raridade de reacção urticariforme imediata. Observou-se eritema imediato não infiltrado com igual frequência em ambos grupos e independentemente do tipo de resposta tardia.

2. Apenas se atribuíu significado às reacçoes tardias fortemente positivas (eritema infiltrado, com pápulas, vesículas e diâmetro superior a 30 mm.), as quais surgiram quáse exclusivamente em formas de tinha muito inflamatórias.

3. A existência de tuberculose pulmonar activa ou de hipersensibilidade tuberculínica não influíu na resposta à tricofitina.

4. A inoculação experimental de dermatófitos só modificou a sensibilidade à tricofitina quando se produziram lesoes muito inflamatórias.

## Acknowledgements

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# SURVIVAL OF LYOPHILIZED YEASTS

Mary E. Burns

*Department of Pathology, Cambridge**

Some members of the genus *Candida* and genus *Saccharomyces* are freeze-dried by a relatively new method, which is described in full. Stability of the desiccates is measured by colony counts and tested by exposure to 100° C. and long term storage at room temperature. An attempt is made to correlate the survival under both conditions.

Yeasts are excellent experimental subjects for investigating theories of freeze-drying. The reasons for this are as follows:

(i) The yeasts' size, which is midway between bacteria and protozoa.
(ii) Morphological and physiological complexity.
(iii) Variety and availability.

There are a few factors to consider when freeze-drying yeasts. The age at which the cultures are dried is important. Old cells are frail. In the *Saccharomyces* the walls of old cells are covered with the scars of their budded offspring; these scars would result in cell wall weakness, which would affect recovery after drying. (Mortimer & Johnson, 1959). Usually yeasts are grown for 24-48 hours at their optimal temperature, and then harvested for lyophilization.

Some yeasts which have a good survival rate on freeze-drying, and are heat resistant in the dried state, have this property of resistance in their natural vegetative state. An example is the genus *Candida*; the pathogenic members of this group are more heat resistant than the others (Miura & Kusunoki, 1954). I found *Saccharomyces fragilis*, earning its name in the natural state, also had a poor rate of survival on freeze-drying and in its heat resistance test. Therefore the basic characteristic of the yeast to be dried should be noticed, for some are more frail than others and may be harder to lyophilize successfully. Faulty resuscitation procedure can also cause low survival rates, for the loss of cell constituents on too rapid rehydration of dried yeasts has been shown by Herrera, Peterson, Cooper & Pepler (1956).

## HISTORICAL

Ever since earliest work in cell preservation, the occasional yeast has been frozen, or dried, or freeze-dried (Macfadyen, 1900 b, c; Elser, Thomas & Steffen, 1935). But the principal reports of entire yeast culture collections being preserved by drying seem to have begun with Wickerham & Andreasen (1942), who examined 400-odd cultures for a period of 2 years. In 1 year 93·8 % of cultures originally dried, survived. They mentioned that the death rate of lyophilized cells should be done, but they themselves did not do it.

In 1946 Wickerham & Flickinger published the follow-up work to this original report. Of 1061 yeasts dried in work extending over a period of 4 years, there was a 98·1 % survival of all yeasts dried when tested at an average time of 2 years. Wickerham & Flickinger found no cultural or metabolic changes in the surviving yeasts; and in fact highly recommend lyophilization as a means of stabilizing characteristics, especially for long-term research projects. Brewer's yeast cultures gave a 99·98 % death rate of the cells, and variation in approximately 50 % of the cultures, when freeze-dried by Atkin, Moses & Gray (1949). Also in 1949 Kirsop lyophilized a variety of yeasts, using a colony count method to estimate the survivals. She showed species differences in recovery. Lindegren, 1949, in his yeast genetics research work uses a drying technique similar to the Sordelli method. His laboratory did no quantitative work, but apparently consider the process quite safe enough to preserve the most intricate of genetical characteristics. Also using Sordelli's method of preservation, over a period of from 1 month to 13 years, Rhodes (1950) found there was little difference in the proportion of viable cultures of 405 isolates at any time interval; and at the end of 13 years she found an 88 % survival of the total number of strains dried.

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* Present address, Naval Medical Research Institute, National Naval Medical Center, Bethesda 14, Maryland, U.S.A.

In 1958 at the Freeze-Drying Symposium in London, Brady (published 1960), reported that she had successfully dried a number of organisms. She watched their stability for over 2 years, and found no qualitative changes by the tests she used. In 1958 Guibert & Bréchot, using a wine fermenting strain of *Saccharomyces cereviseae*, obtained an optimal survival of 40 % by freezing it in 1 minute to −30° C. in beer wort, and beer wort plus sucrose. There were changes traceable by using the bio-assay techniques of vitamin sythesis studies, but the usual reactions used for yeast species identification did not change. Following up their first paper, Bréchot & Guibert (1958) dried their yeasts on iron and glass, and used the decolourization of methylene blue broth tubes as the mode of titrating their survivals. The yeast tested was *Saccharomyces cereviseae* var. *ellipsoideus*. They found the optimal survival was obtained after the preparation to be dried was chilled on glass over a period of 1 minute to a temperature of −30 to −50° C., and drying begun from there.

Rhoades (1958) had observed dried products over a period of 20 years. His yeasts did very well, though he advised that the desiccates be kept at a refrigerator temperature of +4° C. for very long term storage. Ingram (1960) has treated a number of yeast species by freeze-drying, particularly those related to the *Saccharomyces* var. *rucksei*, an osmophilic organism; and measured survival by the colony counting method.

### *Comment on historical*

Though this has been an abbreviated review, the point has been made that freezing and drying of yeasts has been done to a considerable extent. However, it is difficult to co-ordinate the results obtained by these workers, except in the most general way, for the following reasons.

(i) The drying devices vary from worker to worker, as do the drying media. The mechanical technique, the machine, the method of exposing the organisms to the cold, vacuum and desiccation, the time and the efficiency of the exposure, are all factors to take into consideration in freeze-drying. So is the nature of the suspending and reconstituting media.

(ii) The methods of estimating survivals also vary. They are: (a) The specimens, often a large selection of species, are alive or dead at the end of the time of the period tested. (b) The specimens are alive or dead as mentioned, and some indication is made that the survivors have retained their characteristics. (c) Quantitative estimations of survival are made, using the colony count method, or methods somewhat less exact. As the dried yeast cultures are extremely stable at refrigerator and room temperatures, quantitative studies of survivals were not thought likely to produce results.

## Design of Experiments

There is still little idea of the pattern of degeneration of vitality in lyophilized yeast cultures, or of their stability in adverse conditions such as high temperatures. There are no exact measurements of the difference of species in respect to their resistance to the freeze-drying procedure, or of their subsequent survival at room temperature.

In these experiments I hoped to show that the stability of lyophilized yeasts at high temperature can be roughly correlated with their stability over a necessarily short period of time at room temperature, the drying technique and menstrum being constant. Testing was done at boiling point only (Greaves, 1960), and at no other elevated temperatures, as a wider number of organisms could be examined by limiting the investigation in this way.

It was thought that these organisms, the yeasts, would by the nature of their complex cells provide clues to techniques of drying which would lead to eventual success of preservation of much larger and more delicate structures (Burns, 1961).

## Methods and Materials

*Freeze-drying*

(i) Aseptic technique was followed. The ampoules were labelled with glass-marking ink.

(ii) 0·2 ml. of the organism+menstrum mixture was put into 6 mm. × 10 cm. freeze-drying ampoules (Edwards Co. E30/C16), using hand-calibrated long nosed Pasteur pipettes. The ampoules were lightly plugged with cotton wool, and were kept on ice until placed in the freeze-dryer.

(iii) The preparations were placed in the pre-chilled —40° C. aluminium head, in the drying chamber, of the machine described by Greaves, 1960.

(iv) After 30 minutes, pumping was begun, the refrigerator condenser being —55 to —60° C. Pressure reached 0·1 mm. Hg. in less than 15 minutes, and 0·02—0·01 mm. Hg. within 18 hours.

(v) After primary drying (18 hours) the ampoules were removed from the drying head, constricted on a roller over an gas-oxygen flame, and put on the secondary manifold. Storage in a $P_20_5$ desiccator was not done unless the delay from primary to secondary drying threatened to be an hour or more.

(vi) Drying was carried out over $P_2O_5$ for a further 18-24 hours, at room temperature.

(vii) The vacuum reached 0·007—0·001 mm. Hg. The ampoules were sealed off at that pressure, using a cross fire gas flame.

(viii) The desiccates were then stored at room temperature in the dark, except in the case of the 100° C. experiments.

### *Culture and preparation of yeasts for drying*

Optimal temperature of growth, on Sabouraud glucose agar, varied from 25-28° C. for the non-pathogenic *Candida*, the *Saccharomyces* and the *Rhodotorula*, to 37° C. for the pathogenic *Candida*.

When the colonies were well developed, in 48 hours, they were scraped off the plates and emulsified in the drying media. No washing was done.

The suspending media were: (i) 10 % bovine albumin + 5 % sodium glutamate; (ii) 10 % bovine albumin + 5 % dextran; (iii) 5 % peptone.
These media were prepared in normal saline. The final pH was 6-7·4. There were no significant differences in titrations in a survey test to see if there could have been agglutination of the blastospores due to saline.

### *Titration*

Titration of the dried products was by the Miles-Misra technique (1938). Possibly because of the relatively large size of the cells, it was found that 50-dropper and to a lesser extent 30-dropper pipette drops would give confluent growth in 1 dilution, and next to no colonies in the next. Therefore, 0·1 ml. drops from the hand calibrated pipettes were used, 4 drops per plate. The drops were then spread lightly with a large loop. This method gave duplicable results. The survivals are measured in viable units and are not of individual cells, because of the yeasts' property of budding and chaining. A suspension of a yeast culture, microscopically examined, generally showed the cells to be in groups of 1-4.

### *Nomenclature and sources*

The nomenclature in this paper is that of the British National Type Cultures of Yeast and Fungi (1958), and of Lodder & Kreger van Rij (1952).

To be certain of their identification, the yeasts were obtained from recognised sources. My thanks are due to: The National Type Cultures of Yeasts and Fungi, The Low Temperature Research Station, Cambridge, courtesy Dr. James Barnett; Addenbrooke's Hospital Dermatology Clinic, Cambridge, courtesy Dr. C. H. Whittle; The Microbiological Unit of the MRC, Cambridge, courtesy Dr. R. Davies.

The yeasts studied and their sizes were: *Candida albicans* (2-5 × 3-7·5μ), *C. tropicalis* (5-9 × 6-12μ), *C. parapsilosis* (2·5-5 × 4-8μ), *C. intermedius* (2·5-5 × 4·5-7μ), *C. guilliermondii* (2·5-5 × 3, 7, 13μ), *C. pulcherrima* (3·5-6·5 × 4-9μ), *Rhodotorula glutinis* (3-5·5 × 5-9μ), *Saccharomyces cereviseae* (3·5-8 × 5-15μ), *S. fragilis* (2·5-5·5 × 4-9μ), *Schizosaccharomyces octosporus* (5-7·5 × 5-11μ), Cheddar cheese yeast, Ginger beer plant yeast. The latter 2 yeasts were not identified and lay between the size ranges of *Saccharomyces* species.

## Results

### *Accelerated degradation*

(i) Figs. 1-6 show that 1 group of organisms—*C. guilliermondii*, *C. intermedius*, *C. pelliculosa* and *C. parapsilosis* are quite resistant to 100° C. for at least 60 minutes, particularly if dried in bovine albumin plus sodium glutamate. *C. albicans*,

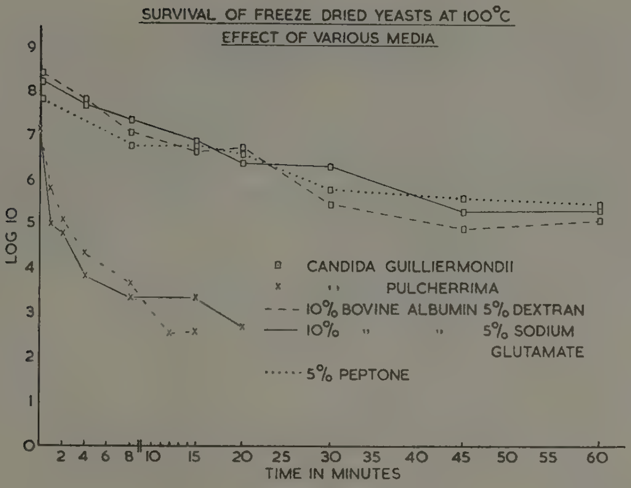

Figure 1.

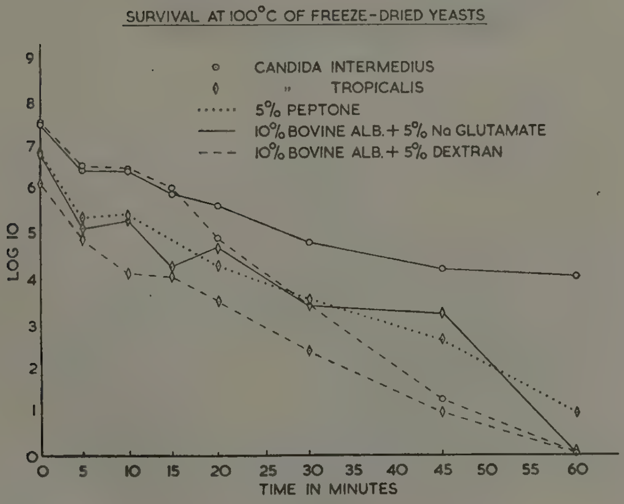

Figure 2.

*C. tropicalis* and *Rhodotorula glutinis* are only moderately so in the same medium. Among the latter group of yeasts are the best known pathogens of the *Candida*; *C. guilliermondii* is known as an occasional pathogen. *C. tropicalis,* though a pathogen, has a somewhat dysgonic growth; it was killed in 60 minutes when dried in bovine albumin plus sodium glutamate, but there was some survival in the peptone desiccate (fig. 2).

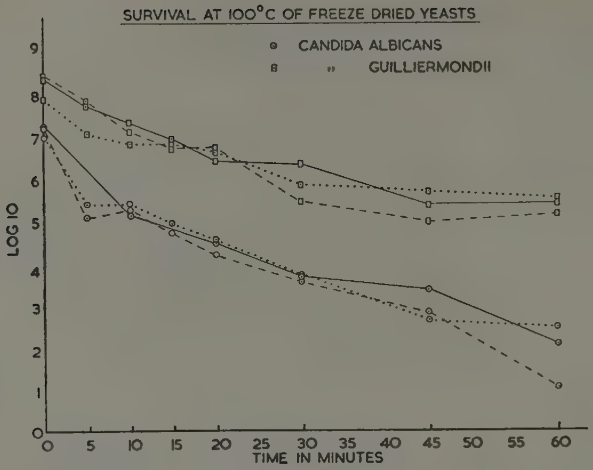

Figure 3.—The key of the media used in drying is the same as in Fig. 1. and Fig. 2.

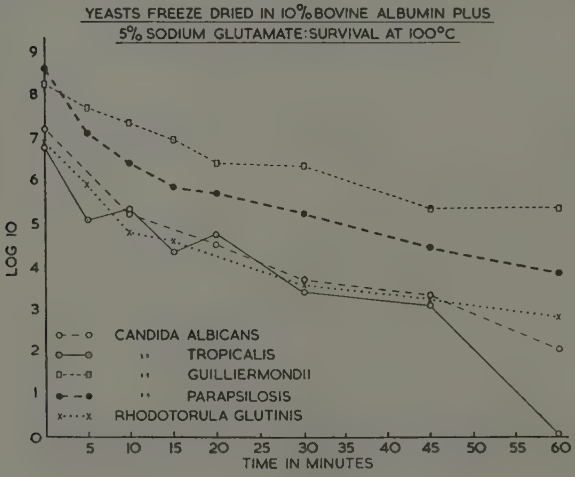

Figure 4.

The performance of these yeasts when compared with the definitely non-pathogenic *Candida*, such as *C. pulcherrima* and *C. mycoderma*, which did not last 30 minutes at 100° C., is in general agreement with the observations of Miura & Kusunoki (1954). They were observing wet preparations on Sabouraud agar slopes, heated to 55° C. and 59° C.; and their test may not have been as severe.

(ii) The Cheddar cheese yeast and the *Saccharomyces* species (figs. 6, 7) lasted less than 30 minutes at 100° C.

(iii) Surviving colonies from long term 100° C. exposure tests were picked directly from the counting plate and examined for variations. There were none, as observed by colony form, microscopy, growth pattern in liquid media, sugar fermentations, or pigmentation.

(iv) Irregularities in yeast survival graphs could be due to simple sampling errors of such an aggregative organism, but the general trend of survival is clear. The yeast most difficult to count was *C. parapsilosis*. It gives a granular, dry, colony growth which is hard to homogenize; therefore its colony counts are somewhat unreliable.

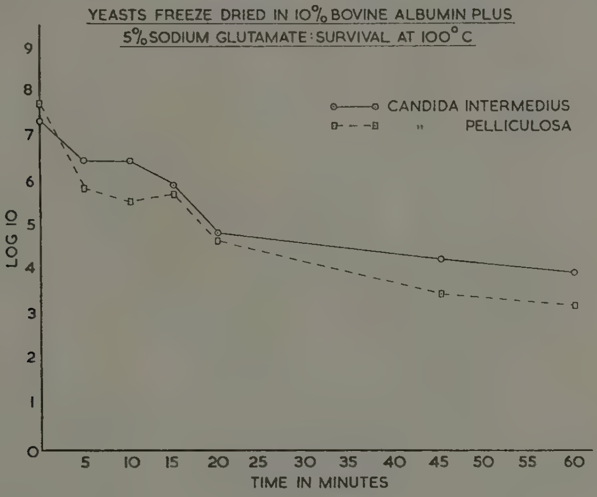

Figure 5.

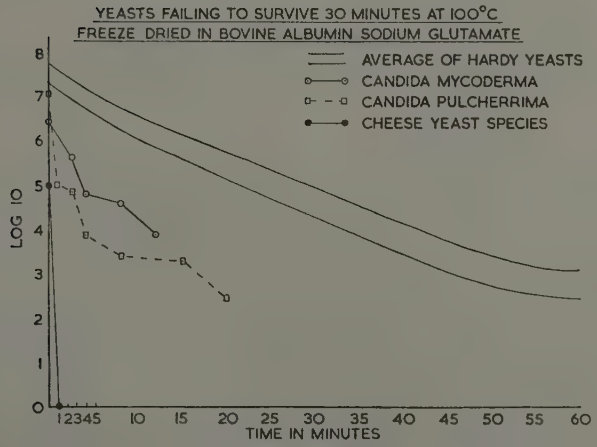

Figure 6.

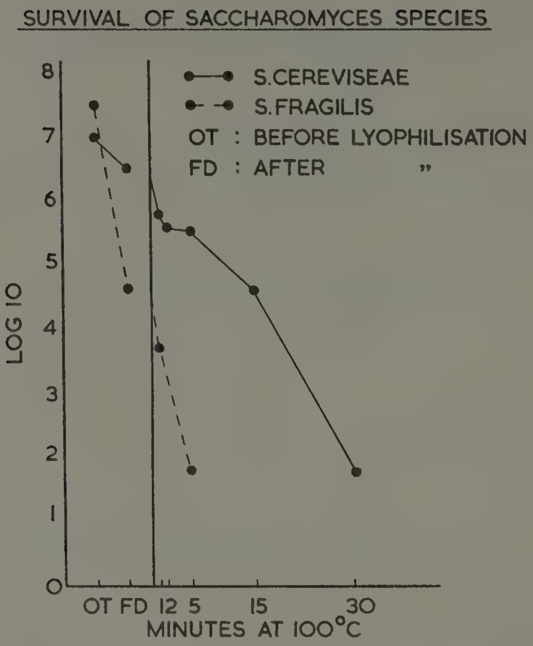

Figure 7.—Dried in 10% bovine albumin + 5% sodium glutamate.

(v) The best preserving media for drying yeasts for exposure to high temperatures rank in the following order (figs. 1, 2, 3).

1: 10% bovine albumin + 5% sodium glutamate
2: 5% peptone
3: 10% bovine albumin + 5% dextran

The last mentioned is more of a drying support for the yeasts than an active biochemical protective agent, but its preservative effect is quite good just the same.

*Some representative room temperature storage results*

The materials and methods were the same as in experiment 1. Storage of the desiccates was at room temperature (20-25° C.) in the dark; and as the months passed ampoules were taken at random from stock, and titrated with the following results.

(i) Organisms which are resistant to freeze-drying tend to show a higher rate of recovery after storage at room temperature over long periods of time; the reverse is also true (fig. 8). Unfortunately not all the titrations immediately after drying were taken, as it was originally planned to compare survival at room temperature with that at 100° C. only.

(ii) The yeasts which were quite stable at room temperature were also quite stable at 100° C. (ex. *C. guilliermondii*) ; and *vice versa* (ex. *C. mycoderma*). A good or

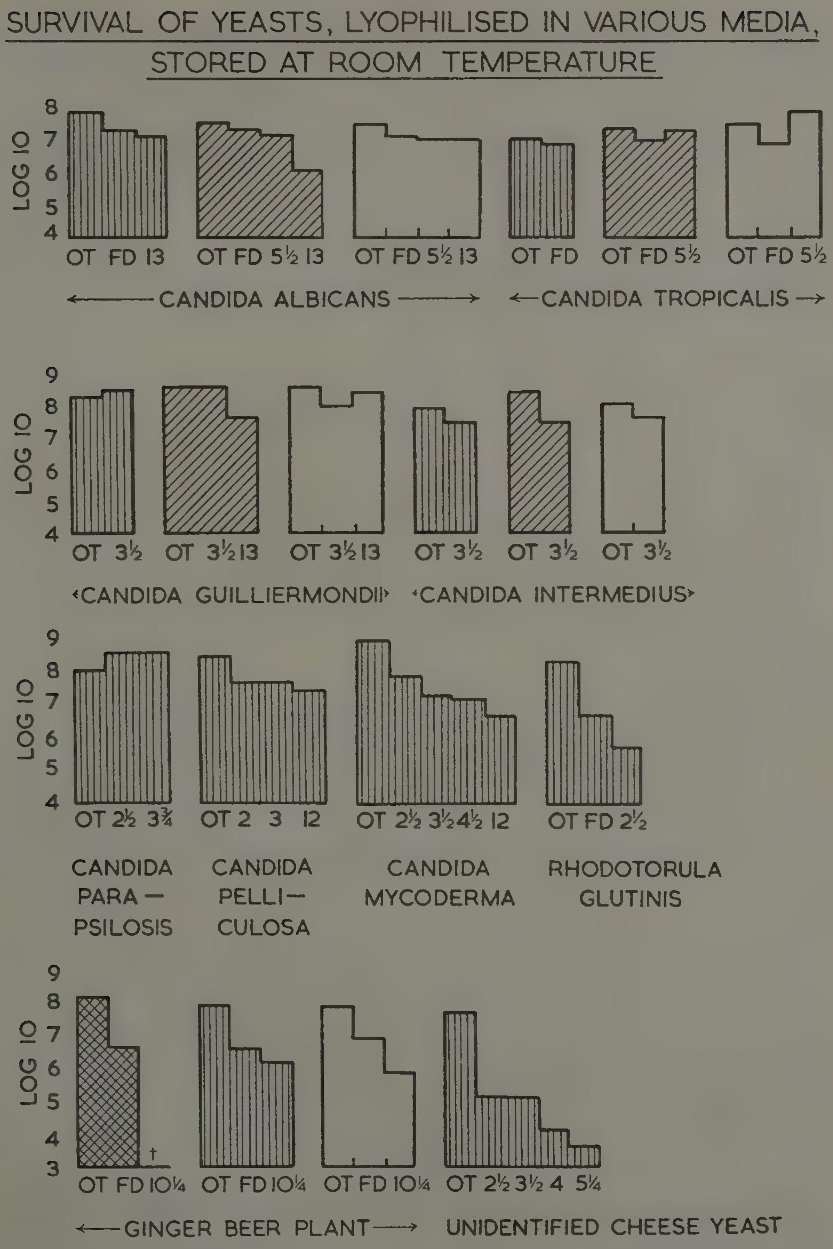

Figure 8.—Times are given in months, ex. 3½, 13. OT=original titre. FD=titre on freeze-drying. ▩ distilled water. ▥ 10% bovine alb. + 5% Na-glutamate. ▨ 10% bovine alb. + 5% dextran. ☐ 5% peptone. † death of specimen.

moderately good survival on drying does not mean the yeast desiccate will be stable at 100° C. An example is *S. cereviseae* (fig 7). *C. tropicalis* shows good stability for 5½ months at room temperature, dried in bovine albumin + sodium glutamate or peptone (fig. 8).

(iii) The differences in menstrua for stabilizing dried materials at room temperature are not so marked as when seen in the 100° C. test. For the times indicated in the histogram of this section, differences in stability in the dried state seem to depend largely on the yeast species themselves.

(iv) When the Ginger Beer Plant was dried in distilled water, it gave an immediate recovery comparable to those when dried in 10 % bovine albumin + 5 % sodium glutamate or 5 % peptone; but it did not survive in storage.

*Yeasts other than the Candida*

(i) *Rhodotorula glutinis* survived freeze-drying with a 1½ log drop in titre, and had a 3 log loss in 1 hour at 100° C., and a 1 log loss in 2½ months at room temperature. Its resistance to freeze-drying and stability in that state are only moderate.

(ii) The Cheddar cheese yeast gave a poor survival in the 100° C. test, and showed a rapid loss of titre at room temperature (fig. 6, 8). Many samples of this yeast were freeze-dried and each showed a 1-3 log loss of titre on drying.

(iii) The Ginger Beer Plant yeast survived freeze-drying with approximately a 1½ log loss in titre, and remained quite stable for 10 months in the dried state. When resuscitated at the end of that time, it produced very good ginger beer.

(iv) The *Schizosaccharomyces octosporus* proved difficult to subculture and to test quantitatively. It seemed to give rather a poor survival on freeze-drying.

(v) The *Saccharomyces* species were heat tested only after drying. *S. cereviseae* lost only ½ log in titre on drying, but had a poor resistance to 100° C. compared to the *Candidae*. *S. fragilis* lived up to its name; it lost 3 logs in titre on drying and within 6 minutes its desiccate was sterilized (fig. 7). This lack of hardiness of *S. fragilis* to drying had no effect on its identifying physical and fermentative characteristics, after it had been grown out in fresh culture.

## CONCLUSIONS

Yeasts tend to behave like bacteria on freeze-drying, in heat tests and on storage (Burns, 1961). However, they are generally frailer, having a more rapid loss of titre in heat tests, and, as far as tested, on room temperature storage; the latter depending on the yeast species. Survival immediately after drying is again comparable to the performance of the bacteria. Some yeast species give little or no loss of titre on drying, and some others are nearly destroyed.

The ultimate factor in the absolute degree of survival seems to depend on the idiosyncracies of the yeast species itself.

It seems possible to test the stability of dried yeasts by accelerated degradation techniques; but at present there are no long term results with which to correlate them.

To set up a collection of dried yeast cultures, the pattern of testing for stability followed by the British N.T.C. of bacteria is a good one with slight modifications as follows: the yeasts are titred before and after drying by the Miles-Misra method; an elevated temperature test is carried out to give a general idea of stability to be expected. Then 1, 3, 6 month and subsequent yearly tests of the desiccates are made, or as many as are indicated by the graphed demonstration of probable continuing loss of viability.

## Résumé

Certains membres des genres *Candida* et *Saccharomyces* sont desséchés sous froid par une méthode relativement nouvelle qui est décrite en détail. La stabilité des préparations ainsi réalisées est évaluée par des comptages de colonies effectués après chauffage à 100° C. et séjour prolongé à la température du laboratoire. L'auteur essaie d'établir une corrélation entre les survies obtenues dans chaque cas.

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# IDENTIFICATION OF *CANDIDA ALBICANS* IN PRIMARY CULTURE

Christine O. Dawson

*Department of Bacteriology, University of Glasgow*

A new technique is described for direct inoculation of swabs from suspected cases of candidiasis. It enables *Candida albicans* to be identified (usually within 24 hrs.) by inspection of the primary culture for chlamydospores. The medium used is Czapek Dox (Oxoid) agar which has not previously been reported to induce chlamydospore formation by *C. albicans*.

The increasing incidence of *Candida albicans* infections in recent years has emphasized the need for a rapid and precise method of identification and one which would eliminate the necessity for isolating the fungus in culture before proceeding to identification would obviously be of considerable advantage. To this end, a number of techniques for identification by colony formation on special media have been reported. Nickerson (1953) suggested that his bismuth sulphite medium, on which *Candida* spp. form typical dark coloured colonies, offered promise for use in isolation of *Candida* spp. from clinical materials. However, Sina & Reiss (1957) found that other organisms gave a similar reaction and concluded that this method of identification was unreliable. Rosenthal & Furnari (1960) reported that Pagano Levine medium (Pagano, Levine & Trejo, 1957-8) was a satisfactory medium for identification of *C. albicans* in primary culture but Sinski (1960) found that a number of strains gave false results. Another method for identification directly by cultural form in primary culture was given by Weld (1953). This consists of inoculating infected material on to Levine eosine-methylene blue (Difco) agar and incubating at 37° C. in an atmosphere of 10 % $CO_2$. However, this method is cumbersome.

Chlamydospore production on special medium is a routine method for identification of pure cultures of *C. albicans* but this method has not, to my knowledge, been previously applied in direct identification. In this laboratory Czapek Dox (Oxoid) agar* has been used for some years as a chlamydospore medium. Using a simple technique for inoculation of this medium with swabs from cases of suspected candidiasis it is possible to identify *C. albicans* by the presence of chlamydospores in the primary culture within 24 hrs. This paper describes investigations which have shown Czapek Dox agar to have a number of advantages over other media and that the swab inoculation technique is suitable for use in routine diagnostic laboratories.

## Materials and Methods

### 1. *Comparison of chlamydospore media*

Twenty seven isolates of *C. albicans* and 14 strains of unidentified mycelial yeasts were tested for chlamydospore formation on Czapek Dox agar, 2 types of corn meal agar and rice infusion agar. The dehydrated Czapek Dox agar and corn meal agar (Oxoid) were re-constituted according to the maker's instructions, a batch of corn meal agar was prepared from freshly ground maize by Benham's method (1931) and the rice infusion was prepared according to Taschdjian (1953). The media were sterilised in universal bottles in 15 ml. lots and when required were melted and poured into fine glass Petri dishes. The test strains were inoculated on to each medium in 2 ways.

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* Czapek Dox agar (Oxoid) is prepared by Oxoid Division, Oxo Ltd., Thames House, London, E.C.4. This medium contains magnesium glycerophosphate without which it is unsuitable for chlamydospore production. Unless stated otherwise, in this paper Czapek Dox refers to the Oxoid product.

(a) *Streak inoculation*

A light inoculum was cut across and through the agar to the glass base of the Petri dish, then the agar to one side of the streak was raised along the length of the streak with the inoculating needle, so that the inoculum was spread between the agar and the glass base of the dish.

(b) *Sandwich inoculation*

A very light inoculum was spread in short parallel streaks on the agar taking care not to break the surface film. This inoculum was covered with a thin (2-3 mm.) sheet (approx. 2·5 cms. sq.) of sterile 2 % water agar cut and transferred from a previously prepared Petri dish with a flamed scalpel.

Cultures were incubated at 28° C. and read unopened through the glass base of the dish by direct inspection with a low-power microscope objective or through the covering agar after removing the lid from the Petri dish.

### 2. *Investigation of factors affecting chlamydospore formation*

(a) *Density of inoculum*

To study the effect of the density of the inoculum on chlamydospore formation a series of dilutions of a pure culture of *C. albicans* were made in a sterile 1 % aqueous solution of the wetting agent Tween 80, reported to enhance pseudomycelium and chlamydospore formation by Seeliger (1955) and Taschdjian (1957). Standard inocula of each dilution, estimated by haemocytometer counts, were made on Czapek Dox agar by the sandwich method and results were read after 19, 24 and 48 hrs. incubation at 28° C.

(b) *Temperature*

Several strains of *C. albicans* were inoculated on Czapek Dox agar by the streak and sandwich methods, incubated at 24° C., 28° C. and 37° C. and examined at 24 hr. intervals for the presence of chlamydospores.

### 3. *Direct identification of C. albicans from swabs*

To investigate the suitability of the agar sandwich method of inoculation for direct identification of *C. albicans* arrangements were made to obtain a supply of vaginal and mouth swabs.

A sterile 1 % aqueous Tween 80 solution was supplemented with penicillin and streptomycin added aseptically to give a final concentration of 200 units/ml. penicillin and 400 units/ml. streptomycin. If stored at 5° C. the antibiotics in this solution remained active for several weeks. Approximately 1 ml. of solution was added to each tube containing a swab and the tube shaken gently. If the liquid became unduly turbid more solution (0·5-1 ml.) was added. Vaginal swabs, which had been found frequently to be heavily contaminated with bacteria, were left in the solution for at least 30 mins. but mouth swabs were inoculated within a few minutes. The sandwich inoculation was made by rubbing the swab gently on the surface of the Czapek Dox agar in a Petri dish and covering the inoculum with a sheet of 2 % water agar. Several inocula separated by channels cut in the basal agar were made on each Petri dish. The plates were incubated at 28° C. and the first reading by direct inspection was made within 24 hrs., in most instances after 18 or 19 hrs. Subsequent readings were made at 24 hr.

intervals. The moistened swab was also used to inoculate a glucose peptone agar slope in the normal way.

The results which were obtained from the sandwich inocula were checked by making streak inoculations on fresh Czapek Dox agar plates from the glucose peptone cultures. A random sample consisting of about half the strains which formed chlamydospores and of all strains in which chlamydospores did not develop in sandwich inocula were checked in this way. In addition, about half the strains in the latter (negative) group were inoculated by the streak method on to rice infusion agar or corn meal agar or both.

## Results

### *Comparison of chlamydospore media*

When read at 24 hrs. chlamydospores had been formed by 23 of the 27 strains of *C. albicans* on Czapek Dox agar, by 21 on rice infusion agar and on corn meal agar (Oxoid) and by 10 on the laboratory made corn meal agar. At 48 hrs., 25 of the 27 strains had formed chlamydospores on both Czapek Dox agar and rice infusion agar, 24 strains on the corn meal agar (Oxoid) and 20 strains on the laboratory made corn meal agar. All 27 strains of *C. albicans* formed chlamydospores on at least 1 of the media. From estimations of the numbers of chlamydospores formed it was apparent that Czapek Dox and rice infusion agars were the most satisfactory media and that the method of inoculation, sandwich or streak, did not significantly affect the result. None of the 14 strains of unidentified yeasts formed chlamydospores on any medium.

### *Density of Inoculum*

Examination of cultures made from inocula of the heaviest suspension which contained approx. $35{,}000 \times 10^7$ cells/ml. showed at 24 hrs. predominantly budding cells with pseudomycelium and chlamydospores confined to the outer edge of the growth. All dilutions between $3{,}500 \times 10^7$ and $50 \times 10^7$ behaved in a similar manner showing well-developed pseudomycelium and numerous well-formed chlamydospores at 19 hrs. It was noted that as the number of cells in the inoculum decreased the number of yeast cells formed also decreased and there was a concomitant increase in pseudomycelial development. At a dilution of $4 \times 10^7$ cells/ml. growth was almost entirely composed of pseudomycelium but the chlamydospores were immature at the 19 hr. reading. Further dilution led to chlamydospore formation being delayed until 48 hrs. At the lowest dilutions (approx. $4 \times 10^2$ cells/ml.) chlamydospores were formed in grape-like clusters on abnormal and stunted pseudomycelium.

### *Temperature*

Chlamydospores formed at both 24° C. and 28° C. but took rather longer to develop at the lower temperature and therefore all subsequent work was done at 28° C. No strain formed chlamydospores at 37° C.

### *Direct inoculation of swabs*

The results of this study are summarized in table 1. *C. albicans* was identified from 120 (75 %) of the 160 specimens investigated and 114 (95 %) of the positives were detected by direct examination of the primary growths in agar sandwich. In 99 (82·5 %) cases the identification was made within 24 hrs., in 10 (8·33 %) within 48 hrs. and in 4 (4·17 %) within 72 hrs. Only 6 (5 %) cases of infection were

| *Site and no. of specimens* | *Specimens from which C. albicans was identified* | | | | | | *Specimens negative for C. albicans (% total specimens)* |
|---|---|---|---|---|---|---|---|
| | *Total number (% of total specimens)* | *Determined by chlamydospore formation in primary cultures from swabs inoculated by the sandwich technique on Czapek Dox agar* | | | | *Chlamydospores not formed in sandwich inocula from swabs but formed in streak inocula of culture from glucose peptone slope (% total +ve specimens)* | |
| | | *Total det. by direct exam. (% total +ve specimens)* | *Within 24 hrs. (% total +ve specimens)* | *Within 48 hrs. (% total +ve specimens)* | *Over 48 hrs. (% total +ve specimens)* | | |
| Vagina 117 | 83 (70·94%) | 77 (92·77%) | 71 (85·54%) | 5 (6·02%) | 1 (1·2%) | 6 (7·23%) | 34 (29·06%) |
| Mouth 43 | 37 (86·04%) | 37 (100%) | 28 (75·68%) | 5 (13·51%) | 4 (10·81%) | — | 6 (13·95%) |
| Total 160 | 120 (75%) | 114 (95%) | 99 (82·5%) | 10 (8·33%) | 5 (4·17%) | 6 (5%) | 40 (25%) |

TABLE 1.—RESULTS OF INVESTIGATION OF SANDWICH INOCULA ON CZAPEK DOX AGAR FOR IDENTIFICATION OF *C. albicans* IN PRIMARY CULTURES FROM SWABS

not detected on direct examination. Of these, 5 formed chlamydospores on Czapek Dox agar inoculated from the glucose peptone culture and the sixth formed only a very few chlamydospores on rice infusion agar. Chlamydospores were not produced on any of the test media inoculated from glucose peptone cultures of the other specimens and it was assumed that *C. albicans* was not present. Of the 117 specimens examined from vagina 34 (29·06%) were negative for *C. albicans*. 33 of these negative specimens showed yeast growth while the other yielded bacteria only. Forty-three specimens were examined from mouth, 6 (13·95%) were negative for *C. albicans* and from 4 of these 6 specimens no organisms were isolated. The remaining 2 gave rise to yeasts other than *C. albicans*.

## DISCUSSION

There can be little doubt that Czapek Dox agar (Oxoid) is comparable in efficiency to other recognised chlamydospore media. Moreover, it has the advantage of being of standard chemical composition and therefore is not subject to the variation of "natural" media such as was found by Gordon, Bradley & Grant (1952) for corn meal agar. However, Czapek Dox agar medium prepared in the laboratory by the orthodox formula is not suitable as a chlamydospore medium. The ingredient which makes the Oxoid product satisfactory for use is magnesium glycerophosphate which may be replaced by Tween 80 without any appreciable loss in efficiency.

The formation of chlamydospores is apparently influenced by a number of factors. Kligman (1950) advocated covering the inoculum with a flamed coverslip (Benham, 1931) to stimulate chlamydospore formation by reducing the oxygen tension. However, reading of the results is difficult as the coverslip becomes opaque due to condensation and a film of water tends to collect below it. The streak and sandwich methods give conditions of growth similar to the above method without the attendant disadvantages.

There are conflicting reports on the effect of moisture on chlamydospore formation. Linossier & Roux (1889) and Shrewsbury (1934) used liquid medium while Marples & di Menna (1952) reported that old medium which had dried out was preferable. With Czapek Dox agar better results were obtained by using freshly poured medium.

This study has shown that the density of the inoculum, by controlling the distance between individual cells, affects chlamydospore formation although there is a wide range of tolerance. At very high concentrations the growth of *C. albicans* consists mainly of yeast cells with a little pseudomycelium at the edge of the colony. Consequently although the chlamydospores are formed within 24 hrs. they are few in number and their distribution is strictly limited. Inocula of extremely low concentrations result in delay in development of chlamydospores until 48 hrs. and they are borne in clusters on extremely scanty and dwarfed pseudomycelia.

This may explain why 4 cultures from mouth were found to be positive only after 48 hrs. In each of these cultures a very light growth of *C. albicans* was noted, in the order of 2 or 3 colonies per agar square and per glucose peptone slope.

Certain cultures from vaginal swabs (6) failed to produce chlamydospores in primary culture. It is tempting to assume that this may have been caused by an over-heavy inoculum. However, in my opinion, a more likely explanation is that excessive bacterial contamination, which the antibiotic treatment did not overcome, prevented the normal development of *C. albicans*. Paine (1952, 1958) notes several instances of bacterial inhibition of *Candida* growth.

A possible objection to the identification of *C. albicans* by the presence of chlamydospores will be that *C. stellatoidea* first reported by Jones, Martin & Durham (1938) also forms them. However the systematic position of this yeast is still in doubt. Lodder & Kreger van Rij (1952) consider it as a distinct species while Winter & Foley (1955) find no reason to justify its separation from *C. albicans*. Until further investigations to clarify this situation are made there seems no reason why chlamydospore formation should not continue to be used as a diagnostic feature for *C. albicans*. Indeed from the study of the few strains of *C. stellatoidea* which I have had available, it would seem that the method in which chlamydospores are formed may well prove to be a distinguishing characteristic for the 2 yeasts. On Czapek Dox agar the chlamydospores of *C. stellatoidea* tend to form in chains (c.f. Lodder & Kreger van Rij, 1952 fig. 216) and require at least 48 hrs. incubation at 28° C. to form.

## RÉSUMÉ

On décrit une nouvelle technique pour la mise en culture directe d'écouvillons provenant de cas suspects de Candidose. Elle permet (habituellement dans les 24 heures) l'identification de Candida *albicans* par l'observation des chlamydospores dans les primo-cultures. Le milieu utilisé est le milieu de Czapek Dox (Oxoid) gélosé. Il n'avait pas encore été mentionné comme pouvant induire la formation de chlamydospores de *C. albicans*.

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# TOLERANCE OF CERTAIN FUNGI TO ACTIDIONE AND ITS USE IN ISOLATION OF GYMNOASCACEAE

H. H. Kuehn and G. F. Orr

*Rohm & Haas Co., Bristol, Pa. and University of California,*
*Los Angeles, Calif., USA*

Vegetative cells of certain Gymnoascaceae were grown in submerged liquid culture with conc. of 1-6 mg./ml. actidione. All species grew well at these levels of actidione with the exception of one strain of *Gymnoascus reessii. Pseudoarachniotus hyalinosporus* grew well in the presence of 8-15 mg./ml. actidione. Ascospores, arthrospores and/or aleuriospores of certain species of Gymnoascaceae were tested for resistance to conc. of 1-6 mg./ml. actidione and they germinated and grew normally. Spores of certain Gymnoascaceae, aspergilli, penicillia and *Coccidioides immitis* were used to prepare a mixed spore suspension which was exposed to levels of 1-6 mg./ml. actidione. *Penicillium humuli* was not inhibited by actidione and overgrew the test plates. Omission of *P. humuli* permitted the species of Gymnoascaceae to be isolated from the mixed spore suspension.

Leach, Ford & Whiffen (1947) gave the first report concerning actidione* (cycloheximide) and indicated its fungistatic properties. Since that time many investigations have been made concerning the action spectrum of this antibiotic. Tukey (1948) reported that actidione was effective against powdery mildews at a conc. of 0·005 mg./ml. Whiffen (1948) determined the influence of actidione in conc. up to 1 mg./ml. on 12 bacteria, 22 yeasts and 14 fungi pathogenic to man. She found that the bacteria were not inhibited at levels of 1 mg./ml. Of the yeasts, 9 spp. were inhibited by 0·00017 mg./ml. while 5 spp. were not inhibited with 1 mg./ml. and the tolerance of the remaining yeasts ranged between these 2 extremes. Eleven fungi (*Candida albicans, Coccidioides immitis, Geotrichum* sp., *Hormodendrum compactum, Nocardia asteroides, Sporotrichum schenkii, Trichophyton rubrum* and 3 strains of *T. mentagrophytes*) were not inhibited with 1 mg./ml. actidione. *Blastomyces dermatididis* was inhibited by 1 mg./ml.; *Monosporium apiospermum* was inhibited by 0·025 mg./ml. *Phialophora verrucosa* by 0·0125 mg./ml. and *Cryptococcus neoformans* by 0·00024 mg./ml.

Vaughn & Hamner (1949) found that 0·002-0·02 mg./ml. actidione inhibited the growth of certain phytopathogens in varying degrees. Schmidt, Alvarez-De Choudens, McElvain, Beardsley & Tawab (1950) reported that actidione was an effective inhibitor of *C. neoformans* in a liquid medium. Whiffen (1950) studied the reaction to actidione of 33 phytopathogenic fungi belonging to the Phycomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes. Her determinations were made upon spore suspensions streaked on agar plates which contained 0·000062 – 0·1 mg/ml. actidione. In 3 instances vegetative mycelium was used rather than spore suspensions. All 33 spp. were inhibited with less than 0·1 mg./ml. except for *Ramularia pastinaceae* and *Fusarium lycopersici* which were inhibited by exactly 0·1 mg./ml.

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* Trade name, Upjohn Co., Kalamazoo, Michigan, USA.

Phillips & Hanel (1950) used actidione to eliminate fungi as contaminants in bacteriological media. They found that 42 strains (27 spp.) of bacteria were not inhibited with 0·1 mg./ml. actidione, but 1 yeast (*Saccharomyces* sp.) and 12 spp. representing 7 genera of fungi (3 *Alternaria* spp., 3 *Penicillium* spp., 2 *Fusarium* spp., *Aspergillus* sp., *Hormodendrum* sp., *Neurospora* sp., and *Rhizopus* sp.) were inhibited with 0·1 mg/ml.

Wallen & Skolko (1951) found that 0·1 mg./ml. actidione prevented growth of *Ascochyta pisi* in pea agar. Kubo, Mizuno, Narita & Hirai (1951) reported that actidione was effective as a growth inhibitor of *T. pedis* (*T. mentagrophytes*). Whiffen (1951) found that 0·0005 mg./ml. and 0·0008 mg./ml. actidione completely inhibited growth of 2 spp. of *Allomyces*.

It remained for Georg, Ajello & Gordon (1951) to demonstrate the value of actidione as a selective agent for isolation of *C. immitis*. Washed spores, not pregerminated spores or mycelium, of *C. immitis* and 15 strains representing 12 other genera were plated on Sabouraud's dextrose agar containing 20 units/ml. penicillin, 40 units/ml. streptomycin and 1 of 3 levels of actidione: 0·1, 0·5 or 1·0 mg./ml. The following strains grew with a level of 1 mg./ml.: *C. immitis, Alternaria* sp., 2 spp. of *Hormodendrum, Penicillium* sp. and *Scopulariopsis* sp. The fungi which were inhibited included 2 spp. of *Aspergillus, Fusarium* sp., *Helminthosporium* sp., *Mucor* sp., *Oospora* sp., *Paecilomyces* sp., *Penicillium* sp., *Rhizopus* sp. and *Trichoderma* sp. Further tests showed only slight inhibition of growth of *C. immitis* with 2-4 mg./ml. actidione.

Walker & Smith (1952) reported that actidione inhibited growth and respiration of *Myrothecium verrucaria*. Furthermore, they found that spores of that fungus were 10 times more sensitive than the vegetative mycelium.

Fuentes, Trespalacios, Baquero & Aboulafia (1952) studied the influence of actidione upon certain saprophytic and pathogenic fungi using Sabouraud's dextrose agar with 0·1 mg./ml. actidione. Of 42 pathogenic fungi, all but 6 strains grew well in the presence of actidione. In general, the 15 strains of saprophytic fungi grew poorly and 6 of them completely failed to grow.

Georg (1953) studied the use of actidione as a selective agent for isolation of dermatophytes from clinical materials. Corke & Chase (1956) used 0·04 mg./ml. actidione to prevent fungal growth in the selective isolation of actinomycetes. Fergus (1956) used levels up to 0·1 mg./ml. actidione in agar media and found only a slight inhibitory influence upon most of the wood-staining fungi tested. Durie & Frey (1957) isolated a new species of *Trichophyton, T. terrestre,* using as a selective medium Sabouraud's dextrose agar containing 20 units/ml. penicillin, 40 units/ml. streptomycin and 0·5 mg/ml. actidione.

Recently, Kuehn, Orr & Ghosh (1961) reported the routine use of Sabouraud's dextrose agar supplemented with penicillin, streptomycin and 0·5 mg./ml. actidione for the isolation and possible maintenance of representatives of Gymnoascaceae. The level of actidione used, 0·5 mg./ml., in general sufficed to reduce the numbers of contaminating fungi encountered in their work. The purpose of the present series of investigations was to determine whether increased levels of actidione could be utilized in order to facilitate the isolation and maintenance of cultures of Gymnoascaceae.

## MATERIALS AND METHODS

As already noted, Walker & Smith (1952) reported spores of *M. verrucaria* to be more sensitive than vegetative tissue to actidione. Therefore, this investigation was divided into 2 parts. The first part concerned the influence of various conc. of actidione on growth of vegetative tissue and the second part involved a series of experiments designed to test the reaction to actidione of ascospores, arthrospores and/or aleuriospores of various species.

### Part I—Vegetative tissue.

One strain each of *Pseudoarachniotus hyalinosporus*, *Eidamella deflexa* and *Myxotrichum uncinatum* and 8 strains of *Gymnoascus reessii* were cultured in shaken flasks in the presence of various levels of actidione. The strains used and their sources were as follows: *Ps. hyalinosporus* 0-846 (0 numbers are accession numbers of G. F. Orr), lung, kangaroo rat (*Dipydomys* sp.), Blythe air strip, Blythe, Riverside County, California; *E. deflexa* 0-233, soil, 12 miles northeast of Mercy Hot Springs, Fresno Co., Calif.; *M. uncinatum* RSA (Rancho Santa Ana Botanic Garden) 56 (NRRL A-3610), dung, Urbana, Champaign Co., Illinois. The strains of *G. reessii* were: 0-135, soil, 8 miles south of Los Banos, Merced Co., Calif.; 0-162, lizard dung, Oildale, Kern Co., Calif.; 0-214, soil, 8 miles south of Los Banos, Calif.; 0-301, lizard dung, 8 miles south of Los Banos, Calif.; 0-304, lizard dung, 12 miles north of New Idria, San Benito Co., Calif.; 0-309 (NRRL A-3612; RSA 60), goat dung, Urbana, Ill.; 0-575, rat dung, 7 miles southwest of Palo Verde, Imperial Co., Calif.; 0-747, soil, 16 miles southwest of Inyokern, Kern Co., Calif.

Stock cultures were maintained on YpSs slants. Small squares of agar containing mycelium were removed from the stock slants and used to inoculate flasks which contained 4% glucose and 1% peptone with 100 ml. medium/500 ml. Erlenmeyer flask. Such flasks are customarily termed 'inoculum' flasks, since they are used to bring up seed inoculum for the experimental flasks. The inoculum flasks were incubated on a rotary shaker at 28° C. for 24 hr. after which they were used to inoculate the experimental flasks. An inoculum of 1% by vol. was used per flask. Experimental flasks were incubated on a rotary shaker at 28° C. for 2½-4 days following which the amount of growth was measured using dry wt. as an index. The procedure followed was the same as that presented in detail by Kuehn & Crosby (1959).

Experimental flasks contained 1 of 3 types of media: Medium 1: 4% soluble starch, 1% glycine, 1 mg% each of adenine, uracil and guanine, 5 mg% xanthine, 0·8 mg% biotin, 0·2 mg.% pyridoxine, 0·2% each of $K_2HPO_4$ and $MgSO_4 \cdot 7H_2O$, pH 7·0.

Medium 2: Same as medium 1 except 1% casamino acids was used in place of glycine.
Medium 3: 1% peptone, 4% maltose, pH 6·8.

A stock solution of actidione was prepared by dissolving it in ethanol and making up to a conc. of 4g/100 ml. with distilled water. This solution was sterilized by Seitz filtration and aliquots were added to the sterile experimental media until the desired final conc. of actidione was attained. Results of preliminary experiments showed that conc. of 0·05-0·09 mg./ml. actidione resulted in little or no decrease in growth. Therefore, higher conc. of 1, 2, 4 and 6 mg./ml. actidione were used to test the experimental fungi. In each experiment control flasks with no actidione were included. Duplicate flasks were inoculated in each test series.

### Part II—Spores.

Several sets of experiments were made in order to test the resistance to actidione of the various types of spores present in the test fungi. Pure cultures as well as mixed cultures were used. In the first experiment (I) spores from the following cultures were used: *Ps. hyalinosporus* 0-877, isolated along with *T. rubrum* from a tinea pedis lesion on a human foot, Los Angeles, Calif.; *A. sulfureus*, lung, pocket mouse, 6 miles southwest of Palo Verde, Imperial Co., Calif.; *Spicaria violacea*, 0-211, soil, 6 miles north of Blythe, Riverside Co., Calif.; *P. humuli* 0-76, soil, Oak Grove Campground, Los Angeles Co., Calif.; *A. niger* USDA TC 215-4247; *C. immitis* UCLAB-M-234, lung, human, Burbank, L. A. Co., Calif.; *G. reessii* 0-135, soil, 8 miles south of Los Banos, Merced Co., Calif.; *G. reessii* 0-135 in mixed culture with *Penicillium* sp.; *C. immitis* UCLAB-M-153, lung, human, Berkeley, Contra Costa Co., Calif., mixed with *A. sydowi*; *Ps. roseus* NRRL 1677 mixed with *Penicillium* sp.; *Gymnoascus* sp. 0-160, soil, 12 miles northeast of Mercy Hot Springs, Fresno Co., Calif. mixed with *Penicillium* sp.; *Myxotrichum* sp. 0-36, soil, 4 miles southwest of Palo Verde, Imperial Co., Calif., mixed with *Penicillium* sp.; *Shanorella spiratricha* 0-880, dung, pack rat, Lake Arrowhead region, San Bernardino Co., Calif., mixed with *Penicillium* sp.; *E. deflexa* 0-249, soil, 6 miles southwest of Avenal on Highway 41, Kings Co., Calif.; and several undetermined species of bacteria.

The *Penicillium* spp. were not identified but one was known to be *P. oxalicum*. The bacteria, aspergilli, penicillia and *S. violacea* were included merely to supply some commonly encountered soil fungi and bacteria. *C. immitis* was included as a control because it is known to be relatively resistant to actidione.

This first experiment (I) was repeated with one difference. *P. humuli* and *A. niger* were omitted for reasons to be explained later. Also, *C. immitis* UCLAB-M-166, lung, pocket mouse, 6 miles southwest of Palo Verde, was substituted for the other 2 strains of *C. immitis* which were not sporulating sufficiently well at the time they were required.

In experiment I the following procedure was used. Spore suspensions were prepared by adding approximately 8-10 ml. sterile water to cultures grown in ½ oz. perfume bottle slants (Wilson & Plunkett, 1949). All cultures were grown on Freezing agar (Kuehn, Orr & Ghosh, 1961) with the exception of *C. immitis* which was grown on Sabouraud's glucose agar. The spores were carefully loosened with a sterile wire loop and the bottles were allowed to stand for 20 minutes. These primary spore suspensions were pooled and brought to a final volume of 250 ml. with sterile water. A 2·5 ml. aliquot of this was brought to 250 ml. with sterile water to make a 1:100 spore suspension.

The procedure followed with *C. immitis* was different. Cultures of this species were frozen and then allowed to thaw partially so that the spores would be wet. This was a safety precaution to prevent them from being spread during handling. Sterile water was then added to the partially thawed cultures and the cultures were shaken. This primary spore suspension was then added to the pool of spores from the other fungi before the vol. was brought to 250 ml.

The final 1:100 spore suspension was spray-inoculated on experimental Petri plates using the technique explained by Kuehn, Orr & Ghosh (1961). For each conc. of actidione to be tested 5 replicate 60 mm. diam. plates were used. The basal medium consisted of 1 % peptone, 3 % syrupy glucose and 2 % agar. Stock solutions of actidione were prepared in the same manner as given by Kuehn *et al.* (1961). The conc. of actidione used were 1, 2, 4 and 6 mg./ml. Control plates lacking actidione were also used. For each conc. of actidione 2 types of media were prepared, 1 containing only actidione (type 1) and the other (type 2) containing actidione, penicillin and streptomycin (PSA medium) in accordance with the work of Georg, *et al.* (1951) and prepared according to Kuehn, *et al.* (1961). The PSA medium was also tested in 5 replicate plates. Thus, for each level of actidione to be tested, there were 10 plates.

In experiment II the plates were not spray-inoculated, but instead the spores were inoculated on to the experimental plates by means of a sterile wire needle. A PSA medium was used with the same range of actidione conc. as in experiment I. Only pure cultures were used for the spore inoculum. The fungi tested in experiment II included certain of the fungi used in experiment I (*Ps. hyalinosporus* 0-877, *E. deflexa* 0-249, *Ps. roseus* NRRL 1677, *Shanorella spiratricha* 0-880 and *Myxotrichum* sp. 0-36) as well as certain additional cultures included *Arthroderma tuberculatum* NRRL 2853, isolated from the feathers of a robin, Urbana, Illinois; *M. uncinatum* 0-910, rat dung, Warner Ranch, Riverside Co., Calif.; *G. reessii* 0-932, lizard dung, Borrego Valley Narrows, San Diego Co., Calif.; *G. brevisetosus* 0-608, lung, kangaroo rat, 8 miles north of Palmdale, Los Angeles Co., Calif.; *M. thaxteri* 0-30, lung, kangaroo rat, Blythe, Riverside Co., Calif.; *C. immitis* UCLAB-M-131, soil, in sheep pasture north of Newman, Stanislaus Co., Calif.

Different types of spores were tested in this experiment because of the stage of growth of the cultures. The strains of *C. immitis* form only arthrospores, the culture of *M. uncinatum* 0-910 had not yet formed ascospores and therefore only aleuriospores of this species were tested and the remaining species of Gymnoascaceae produced ascospores which were tested.

The inoculated Petri plates were incubated for 10 days at room temperature (22-24° C). They were periodically examined to determine the rate and sequence of appearance of colonies of the various species.

## Observations and Results

### I—*Vegetative tissue*

Figures 1 and 2 present the results of an experiment in which 8 strains of *G. reessii* were grown in the presence of various levels of actidione. Since most of the strains were incubated for approximately the same period of time, dry weights representing the amount of growth may be compared directly. However, strain 0-135 was allowed to incubate for a slightly longer period of time and therefore the absolute dry wt. appears high when the control results are compared with other strains. However, the relative growth at each conc. of actidione may be compared among all strains. Strain 0-309 was found to be extremely slow growing in these and other experiments not reported at this time. Hence, the incubation time was extended to 6 days. Even after this time, however, no growth occurred in the presence of even the smallest conc. of actidione.

Another factor which must be taken into consideration in comparing growth among these 8 strains is the type of medium used. For growth of strain 0-135 Medium 1, glycine (see Methods section) was used, but for the other strains Medium 2 (casamino acids) was employed. In certain nutritional experiments conducted concurrently with this present work, but not reported here, it was found that for most of the strains casamino acids were a better source of nitrogen than glycine.

It is interesting to note the rather wide variety of reactions of the 8 strains of *G. reessii* to actidione. All isolates, except 0-309, grew well in the presence of 1 mg./ml. Strains 0-301, 0-304 and 0-747 grew better with 1 mg./ml. actidione than in the complete absence of this compound. Strain 0-162 grew nearly as

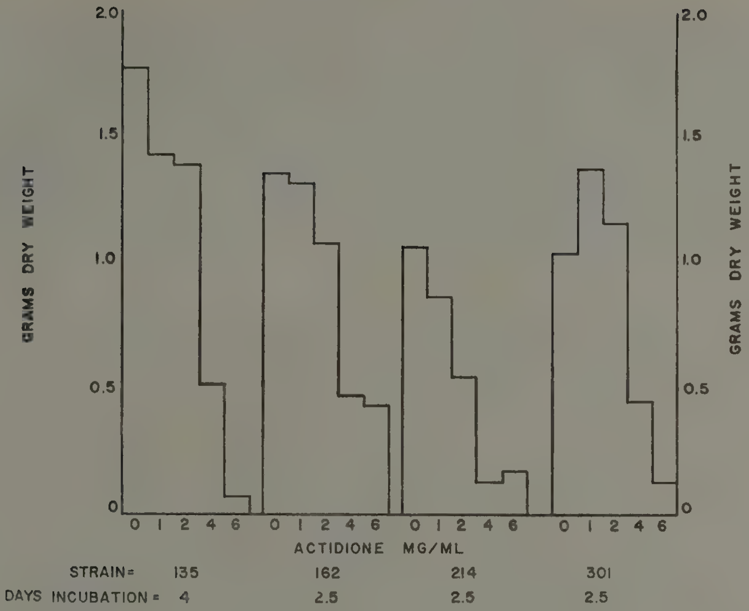

Figure 1.—Growth of 4 isolates of *Gymnoascus reessii* in the presence of actidione.

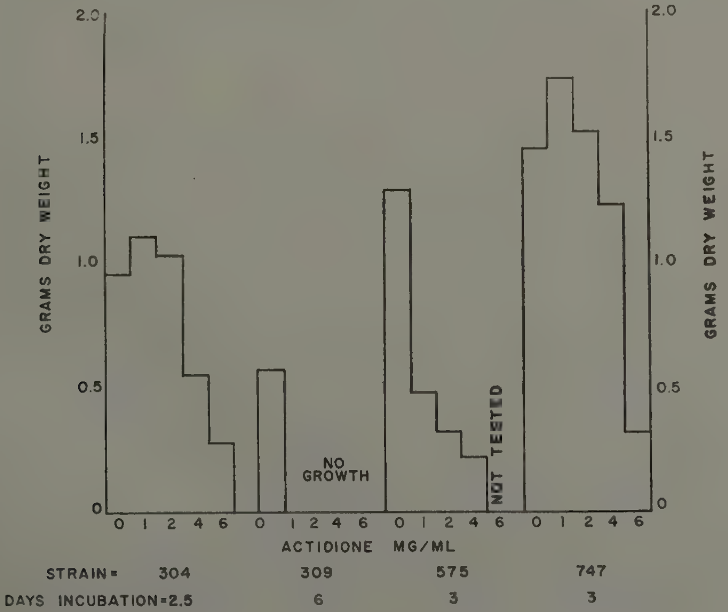

Figure 2.—Growth of 4 isolates of *Gymnoascus reessii* in the presence of actidione.

well in the controls as with 1 mg./ml. Strains 0-135 and 0-204 grew slightly better in the absence of actidione than with 1 mg./ml. Strain 0-575 reacted rather differently since growth with 1 mg./ml. was about one-third that of the controls. Growth curves for strains 0-301, 0-304 and 0-747 had the same shape since growth with 2 mg./ml. actidione was less than with 1 mg./ml. but greater than in the controls. Strains 0-135, 0-162, 0-214 and 0-575, in general, were similar and growth in these strains decreased with increased conc. of actidione.

All strains grew to some extent in the presence of 6 mg./ml. with the exception of strain 0-135, which was able to grow only to a very limited extent, and strain 0-309, which failed to grow in the presence of actidione. Strain 0-575 was not tested at this conc.

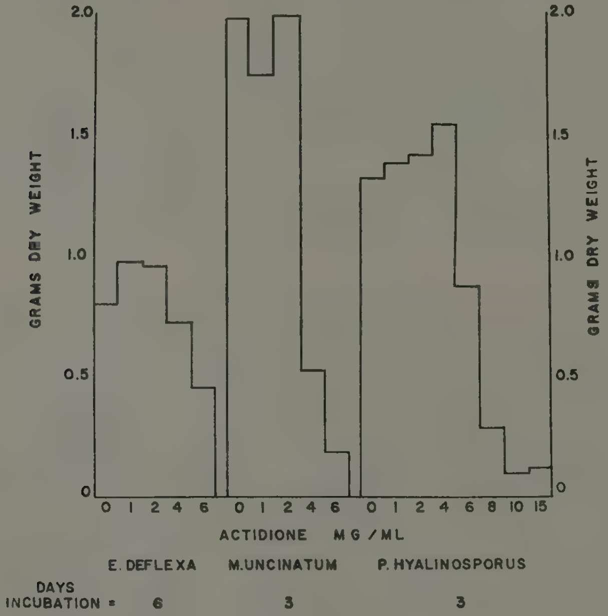

Figure 3.—Growth of certain Gymnoascaceae in the presence of actidione.

Of the 8 cultures, strain 0-747 evinced the greatest absolute amount of growth. In several strains the dry wt. in the presence of 1 mg./ml. actidione exceeded that of the controls.

Further experiments were performed with 3 other spp. of Gymnoascaceae and the results are shown in Fig. 3. Medium 3 (see Methods section) was used as the experimental medium. Results demonstrated that *E. deflexa* 0-233 tolerated actidione to about the same degree as most of the 8 strains of *G. reessii*. Growth in the presence of 1-2 mg./ml. actidione was better than in its absence but growth decreased with 4-6 mg./ml. *M. uncinatum* RSA 56 was able to synthesize a large amount of tissue in the controls and with 1-2 mg./ml. actidione, however, the dry wt. decreased sharply in the presence of 4-6 mg/ml. *Ps. hyalinosporus* 0-846, on the other hand, grew extremely well at all conc. from 1-6 mg/ml. actidione. The absolute dry weights with 4 mg/ml. as well as with 6 mg./ml. were significantly greater than the tissue yield of all other strains at corresponding conc. Therefore, this strain was tested at even higher levels of actidione. Good growth resulted with 8 mg./ml. and some growth occurred even in the presence of 10 and 15 mg./ml. actidione.

### II—*Spores*

In experiment I, as explained previously, spores of both *P. humuli* and *A. niger* were included along with the other fungi and bacteria. *P. humuli* proved to be resistant to all tested levels of actidione and as such quickly overgrew all the plates which contained actidione and in general obscured other slower-growing fungi which subsequently developed. Both Type 1 and Type 2 control plates were covered with *A. niger*, but this fungus did not appear on plates containing actidione. Bacteria grew on all plates of Type 1 (lacking penicillin and streptomycin) and they were present at all conc. of actidione. With increased incubation time the bacteria were overgrown by *P. humuli* or *A. niger*.

For the reasons noted above this experiment was repeated with the same conditions and test fungi except that *P. humuli* and *A. niger* were excluded. Following several days of incubation bacteria were present as before on all media which lacked penicillin and streptomycin (Type 1). Several spp. of *Penicillium* and *Aspergillus* appeared on controls of both Type 1 and Type 2. The following results were noted on Type 1 plates. With 1 mg./ml. actidione, *A. sulfureus* and *S. violacea* appeared on some, but not all, of the plates. With 4 mg/ml. one colony of *A. sulfureus* appeared on one of the 5 replicate plates. *Ps. hyalinosporus* 0-877 appeared latently on one of the control plates. *C. immitis* was not in evidence on any control plates and none of the penicillia or aspergilli appeared on Type 2 plates. From the results of this experiment it was evident that each test species of Gymnoascaceae appeared on at least some of the plates at each of the test levels of actidione. The failure of each of the test fungi to appear on all plates at each conc. of actidione can be attributed to several causes, one of which is the slow growth of most of these fungi. The inoculum contained many organisms with different growth rates so that the slower growing forms were not easily seen. An endeavor was made to subculture each colony on freezing agar in order to identify it. Undoubtedly, slower growing strains were overlooked and therefore were not included in the final tabulation.

Experiment II was prepared in a manner similar to experiment I using two groups of plates, one containing actidione and the other penicillin, streptomycin and actidione. However, the spray inoculation technique used before was not employed. Rather, the test fungi were inoculated on to the plates in triplicate by use of a transfer needle. All the tested fungi grew on all 3 replicate plates at all concentrations of actidione (1-6 mg./ml.) with 2 exceptions. *Shanorella spiratricha* 0-880 and *G. reessii* 0-932 grew on only 2 plates at levels of 6 mg./ml. actidione. Failure of growth on 1 plate may have been due to faulty technique in handling, rather than to actual toxicity.

In the presence of actidione *Ps. hyalinosporus* 0-877 initially was the most rapidly growing species and it attained a colony diam. of 12 mm. in 10 days. Gametangial initials and some asci were present after 10 days incubation. Although the initial growth was less rapid than that of 0-877, *G. brevisetosus* 0-608 grew more rapidly later so that the colonies were finally about 15 mm. diam. after 10 days. Most of the tested fungi formed colonies of 3-6 mm. diam. in 10 days. *G. reessii* 0-932 was the slowest growing strain and attained only one mm. diam. in 10 days.

*E. deflexa* 0-249 normally produces a red pigment in a glucose-peptone medium. However, in the presence of actidione this pigment was much more intense than usual. With this single exception, there were no macroscopic differences between cultures when grown in the presence of actidione or in its absence.

Certain morphological peculiarities were noted microscopically in the arthrospores of *C. immitis*. At all conc. of actidione below 6 mg./ml. strain UCLAB-M-234 produced arthrospores which separated in the normal fashion. However, with 6 mg./ml. the colonies were buff-tan in color and somewhat powdery in appearance. Furthermore, the arthrospores did not disarticulate. Colonies of *C. immitis* UCLAB-M-131 appeared as white hyphal tufts in the presence of 2, 4 and 6 mg./ml. actidione. The hyphal branches on which the arthrospores were formed tended to become distorted, aborted and somewhat curled in the presence of 6 mg./ml. However, the arthrospores formed were normal in their morphology.

## Discussion

It might seem from the results obtained that when certain fungus spp. resistant to actidione, such as *P. humuli*, were present that species of Gymnoascaceae and *C. immitis* could not be isolated from natural habitats. However, one generally would not expect to find resistant forms, such as *P. humuli*, present in such high conc. as used in this study. Thus, with proper dilution of materials it should be possible to isolate gymnoascaceous fungi in the presence of other, faster growing fungi resistant to actidione.

Huppert & Walker (1958) tested 84 strains of *C. immitis* and found that all were resistant to 0·5 mg./ml. actidione. Of these strains, 4 were severely inhibited by 2·5 mg./ml. In our study 3 strains of *C. immitis* grew well in the presence of 6 mg./ml. actidione thus confirming the high tolerance of this species to the antifungal agent.

Previously reported experiments of the tolerance of fungi to actidione were not extended to include the high conc. used in the present study. To our knowledge, therefore, *Ps. hyalinosporus* is the only fungus known to grow well in the presence of 8 to 15 mg./ml. actidione. It would be interesting to extend such studies to include other fungi as well as higher conc. of this chemical.

Spores of *M. verrucaria* have been reported to be less resistant to actidione than the vegetative cells (Walker & Smith, 1952). This does not seem to be true among the gymnoascaceous spp. studied. Growth of both vegetative cells and spores took place at all conc. of actidione which were tested, from 1 through 6 mg./ml. Perhaps a difference in tolerance of vegetative cells and spores could be detected by using higher levels of the chemical. However, for the practical purposes of isolation of gymnoascaceous fungi from nature, a level of 4-6 mg./ml. actidione usually is sufficient since spores and vegetative cells appear to tolerate this conc. of actidione well. Perhaps *P. humuli* would also be inhibited at higher conc. of actidione while most of the Gymnoascaceae would not.

## Zusammenfassung

Vegetative Zellen bestimmter Gymnoascaceen wurden in submersen Kulturen mit einem Zusatz von 1-6 mg./ml. Actidion gezüchtet. Alle geprüften Arten, mit Ausnahme eines Stammes von *Gymnoascus reessii*, wuchsen bei dieser Actidion-Konzentration gut. *Pseudoarachniotus hyalinosporus* wuchs gut bei einem Gehalt von 8-15 mg./ml. Actidion im Nährboden. Ascosporen, Arthrosporen und/oder Aleuriosporen bestimmter Gymnoascaceenarten wurden auf ihre Resistenz gegenüber einer Konzentration 1-6 mg./ml. Actidion getestet, sie keimten und wuchsen normal. Die Sporen bestimmter Gymnoascaceen, Aspergillen, Penicillien und *Coccidioides immitis* wurden gemischt und als Sporensuspension Konzentrationen von 1-6 mg./ml. Actidion ausgesetzt. *Penicillium humuli* wurde nicht durch Actidion gehemmt und überwuchs die Testplatten. Bei Weglassen von *Penicillium humuli* konnten die Gymnoascaceenarten aus der Sporensuspension isoliert werden.

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# MADURA FOOT IN INDIA CAUSED BY *CEPHALOSPORIUM INFESTANS* SP. NOV.

M. L. Gaind

*Department of Dermatology, Military Hospital, Wanowree, Poona, India*

A. A. Padhye & M. J. Thirumalachar

*Hindustan Antibiotics Research Centre, Pimpri, near Poona, India*

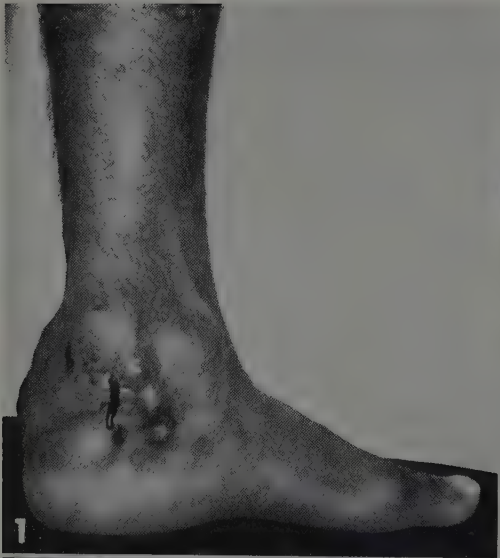
Madura foot caused by a new species of *Cephalosporium* is described. It develops black granules in the host, and in artificial culture forms greyish-white to tan coloured colonies, with fasciculate aerial hyphae in the centre. It differs from *C. madurae* and *C. granulomatis* in the size of conidia and is designated *C. infestans*.

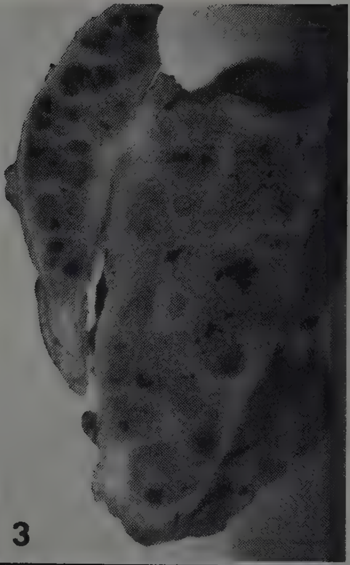
Maduromycosis is a chronic fungus disease usually confined to the foot, but occasionally developing on hand and others parts of the body, characterised by swelling and deformity of the affected parts and also development of sinuses from which serosanguineous fluid oozes out containing numerous granules. The colour of the granules is characteristic of the particular fungus which incites the disease. As many as 26 species of fungi have been reported to cause maduromycosis, of which in U.S.A. *Allescheria boydii* Shear is considered to be of importance from pathogenic point of view. However in recent years, species of *Cephalosporium* are becoming recognised as important incitants of madura-foot, and much attention has been paid to the study of this genus. As already mentioned in a previous paper (Padhye, Sukapure & Thirumalachar, 1961) 3 species of *Cephalosporium* were previously known as incitants of madura-foot; *C. falciforme* Carrion, *C. recifei* Leão & Lobo and *C. granulomatis* Weidman & Kligman. The fourth species to be added from a case of madura foot in Poona was *Cephalosporium madurae* Padhye, Suka, & Thirum. Another case, of arthritis, due to infection by *C. rosea-griseum* Saksena, was reported from Mayo Clinic, Rochester, Minnesota, U.S.A. by Ward *et al.* 1961 who showed that the fungus might have gained entry into the patient through injury (palm thorns) and resulting in hydrathrosis of the knee. This was the first report of arthritis of a major joint due to a fungus. Treatment with amphotericin B for 22 days period proved of no avail. In the present paper, an account of yet another new species of *Cephalosporium* isolated from a case of madura foot in Poona is reported. This species also produces black granules in the host like *C. madurae* but shows differences in morphology and colony characters.

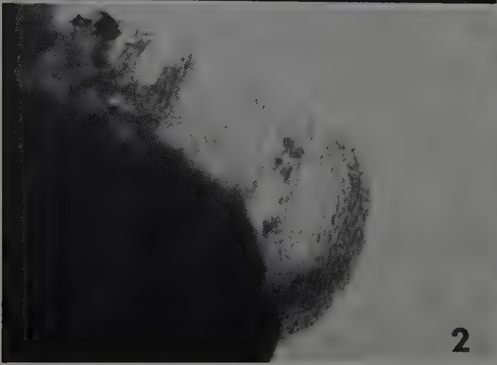
## Observations

### *Case History*

The patient, a 29-year-old soldier in good general health, was admitted to the hospital in March 1959, with a painful swelling on the medial aspect of the left ankle of 6 months duration. The swelling was firmly adherent to the skin,







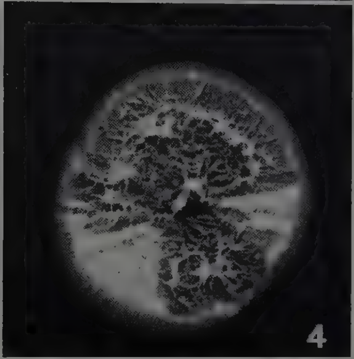


Figure 1.—Photograph of the foot showing multiple draining sinuses.
Figure 2.—Teased preparation of the black granules showing hyphae × 500.
Figure 3.—Excised portion of the infected area of the leg showing the sinuses with granules.
Figure 4.—Culture of *C. infestans* on Sabouraud agar.

*To face page* 231)

but not to the underlying structures. There was a small indolent ulcer on the lower part of the swelling. There was no oedema of the foot or any gland enlargement and x-rays showed no bone involvement. The swelling was incised by the surgeon who noticed large numbers of black granules in the swelling and the case was transferred to the skin department.

Previous history dates back to 1947 when the patient sustained an injury at the left ankle. This was followed by the appearance of a swelling (apparently a Haemotoma) which was incised. The patient was discharged fit from the hospital in 3 months, and was sent to a field area where he sustained another injury on the same spot by a stone. This developed into a small swelling that lasted for a week to 10 days. The patient remained fit until March 1958 when he noticed a small swelling appearing on the inside of the left ankle, but did not report about it for about 8 months. But the swelling gradually increased in size and became painful, and a small ulcer manifested on the lower part of the swelling, when he reported back to the hospital.

### *Treatment*

During the stay of the patient in the hospital, he was treated with large doses of penicillin, sulfa drugs, synermycin and griseofulvin without any marked beneficial effect. Potassium iodide in increasing strength was given up to 100 grains a day for 4 weeks with slight improvement. Finally the whole involved area was excised followed by skin grafting. On operation it was seen that the fungus (black granules) was affecting the skin and subcutaneous tissue (fig. 3); tendons and bone were not affected by the invading fungus. The affected areas have since completely healed after skin grafting except a small area on the outer aspect of the ankle.

### *Mycological investigations*

The black granules were washed with several changes of sterile distilled water. They were 0·3-2·5 mm. diam. Microscopic examinations of the granules in 10% KOH solution showed central mass of compact mycelial strands and chlamydospores. The peripheral region of the granules revealed slender, hyaline branched hyphae, 1-1·5 μ diam. (fig. 2).

Granules were then surface sterilized and were cultured on Sabouraud's glucose agar. Cultures were incubated at 28° C. and 37° C. The growth on Sabouraud's glucose agar was fairly rapid reaching 15-18 mm. diam. in 5-7 days. From more than 300 granules cultured, the same fungus has been found in all instances.

Young colonies on Sabouraud's glucose agar were cottony, dirty white in colour, with blackish periphery. Edge of the colony was smooth, continuous, with deep radiating furrows extending from the centre to the periphery. Central region of the colony showed prominent aerial hyphal strands grouped as erect synnemata (fig. 4). No diffusible pigment was seen. The reverse of the colony was greyish black in colour. Mature colonies of 4-5 weeks, were brown to tan in colour.

Microscopic examination of the colony showed hyphae to be slender, hyaline, branched, sparingly septate and vacuolated, 0·75-1·5 μ diam. Conidiophores were erect, non-septate, unbranched, broader at the base, tapering at the apex, 30-60 × 0·75-1·5 μ. Sporeheads were globose, containing 7-18 conidia held in gelatinous matrix which dissolved in water liberating the spores. Mature conidia were oblong, obovate, straight (noncurved), one-celled rounded at both ends, 3-5 × 1-1·5 μ. Young conidia frequently showed refractile globules at both poles. No terminal or intercalary chlamydospores were seen.

## Discussion

Regarding the identity of *Cephalosporium* species inciting madura-mycosis, a comparative account has already been given (Padhye, Sukapure & Thirumalachar 1961). *C. recifei* Leão & Lobo, and *C. falciforme* Carrion produce white granules, while *C. madurae* Padhye, Suka, & Thirum, develops black granules in the host. Colour of the granules in *C. granulomatis* Weidman & Kligman is not mentioned. *C. madurae* also forms black granules like the fungus under study, but differs in the size of conidia. The conidia in *C. madurae* measure 3-9 μ in length as against 3-5 μ in the fungus under study. *C. granulomatis* is closely related and resembles in colony characters, but the conidia are slightly shorter measuring 2·8 to 3·5 μ. The fungus under study is presented as a new species with the name *Cephalosporium infestans* Gaind & Thirum. Type culture No. H.A.C.C. 114 deposited in Commonwealth Mycological Institute, Kew, England; American Type Culture Collection, U.S.A., Centraal Bureau voor Schimmelcultures, Baarn, Netherlands and Herb. Crypt. Ind. Orient., New Delhi.

**Cephalosporium infestans** Gaind & Thirum. sp. nov.

Coloniae in agaro Czapeck dicto, gossypinae, sordide albae, evadentes cremeae aetate provecta, ambitu nigro. Coloniae distinctae sulcis altis radiantibus, parte centrali monstrante filamenta hyphalia aggregata ut synnemata erecta, pigmento diffusibili nullo. Conidiophori erecti, 30-60 μ. Conidia ovata vel ellipsoidea, hyalina, levia, magnit, 3-5 × 1-1·5 μ.

*Hab*. Induct. 'Pedem Madurensem' in homine, ad Poona atque producit granulos nigros.

*Typus* HACC. 114.

Colonies on Czpek's agar circular slow growing with dirty white submerged and aerial mycelium. On glucose yeast agar colonies are flat, moist, with deep radiating furrows, on Sabouraud's glucose agar and potato-dextrose agar growth moderate, dirty-white, submerged mycelium with whitish aerial ropy strands in central disc forming symemata. Peripheral region of the colony greyish black in colour. No diffusible pigment present. Conidiophores erect, simple, non-septate, broad at the base and tapering at apex, 30-60 μ long. Conidia pleurogenous ovate to ellipsoid, one-celled, 7-18 spores held in mucous mass into spore heads, mature conidia obovate, rounded at both ends, hyaline, one-celled, thin walled, smooth 3-5 × 1-1·5 μ.

*Hab*. Isolated from Madura foot of a patient. Armed Forces Medical College, Poona, India, March 1959.

## ZUSAMMENFASSUNG

Eine Maduromykose des Fuβes durch eine neue *Cephalosporium* Art wird beschrieben. Im Gewebe des Wirts entwickeln sich schwarze Drusen. Der Pilz bildet auf künstlichen Medien grauweiβe bis bräunliche Kolonien mit bündelförmigen Hyphen im Zentrum. Durch seine Conidiengröβe unterscheidet er sich von *C. madurae* und *C. granulomatis* und wird als *C. infestans* bezeichnet.

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# OCCURRENCE OF CALCINOSIS IN THE COURSE OF EXPERIMENTAL FUNGUS INFECTIONS*†

Z. T. Mankowski

*Department of Medicine, Division of Dermatology, University of California Medical Center, Los Angeles 24, California*

During the prolonged presence of *Candida albicans* and *Scopulariopsis brevicaulis* in the spleen of Wistar rats, subcutaneous deposits of calcium salt were observed. The condition is similar to one known as calcinosis in human pathology. Its circumscribed and universal forms were observed. One case of metastatic ossification has been seen.

Calcinosis is a pathological condition characterized by the deposition of calcium salts in the subcutaneous tissues. If this process is limited in its localization it is called calcinosis circumscripta. If the deposits of calcium are observed in other organs it is called calcinosis universalis (Steinitz, 1931). Rothstein & Welt (1936) think that this division is artificial since the 2 forms are probably different stages of the same process. Since the etiology of calcinosis is unknown its occurrence in the course of experimental fungus infection is of interest. Although calcinosis is a rare pathological condition, its etiology is of interest because of its frequent association with diseases of greater frequency and greater clinical significance (Hunter, 1913, Lewandowsky, 1905, Neuwirth, 1906).

## MATERIAL AND METHODS

Sixty adult Wistar rats were operated upon under Nembutol-ether anesthesia and were injected in the spleen with $2{\cdot}5 \times 10^7$ cells of *Candida albicans*. Using the same procedure and the same method of inoculation 40 adult Wistar rats were injected with $1{\cdot}5 \times 10^7$ spores of *Scopulariopsis brevicaulis*. Both organisms were isolated from a human source and were maintained on Sabouraud's agar. The animals were kept on Purina chow diet and received water *ad libitum.*

## RESULTS

All animals which died or were sacrificed were autopsied. Tissues were fixed in formalin, imbedded in paraffin, sectioned and stained with haematoxylin and eosin. Detailed observation of pathological changes will be presented later. On 2 occasions in the animals infected with *C. albicans* nodules were seen on the skin of their backs. The time of their first appearance is difficult to state exactly, because these types of changes were not expected. When they were first observed at the seventh and eleventh month of infection they were already of fair size. As the observation continued the nodules increased slowly in size. At the time of sacrificing the animals, they were of the size $1\frac{1}{4} \times 1\frac{1}{4} \times 1\frac{1}{4}$ cm. The skin over these nodules was partially depilated and it could not be moved over the nodules. The nodules were of hard consistency and could be moved freely over underlying tissues. In 1 case there were several small nodules in a

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* Supported by a grant from the National Institutes of Health Mycology Training—2E-52.

† Some of the material here was presented at the annual meeting of the American Mycological Society in the paper entitled: 'Chronic Pathogenecity of Candida Albicans', on August 28, 1961 at Purdue University, Lafayette, Indiana.

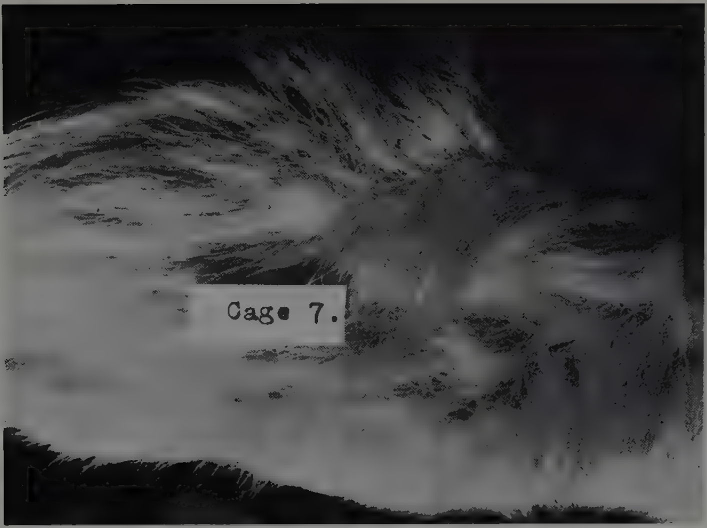

Figure 1.—Nodule in the skin of the back of a rat.

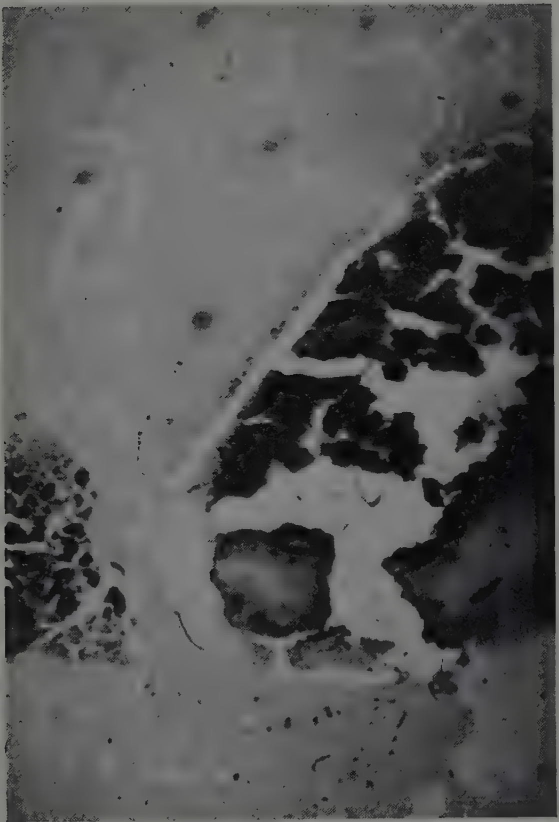

Figure 2.—Calcium deposits in subcutaneous tissues × 75.

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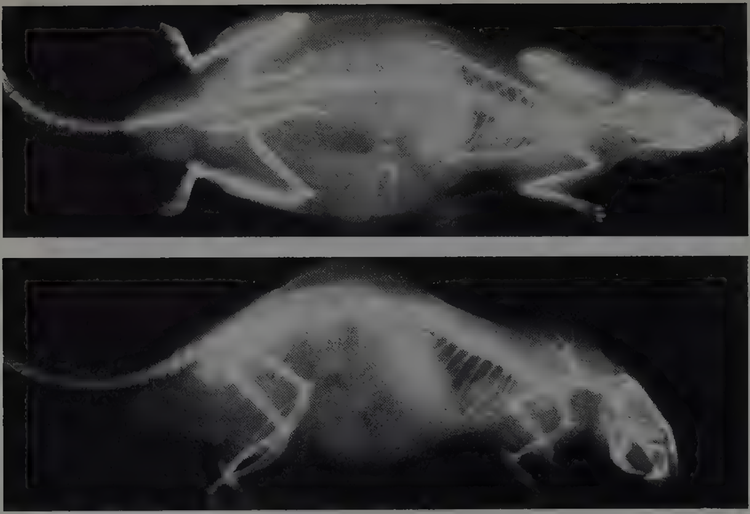

Figure 3.—X-ray picture of a rat showing large subcutaneous calcium deposits. Note the many smaller ones in the abdominal cavity.

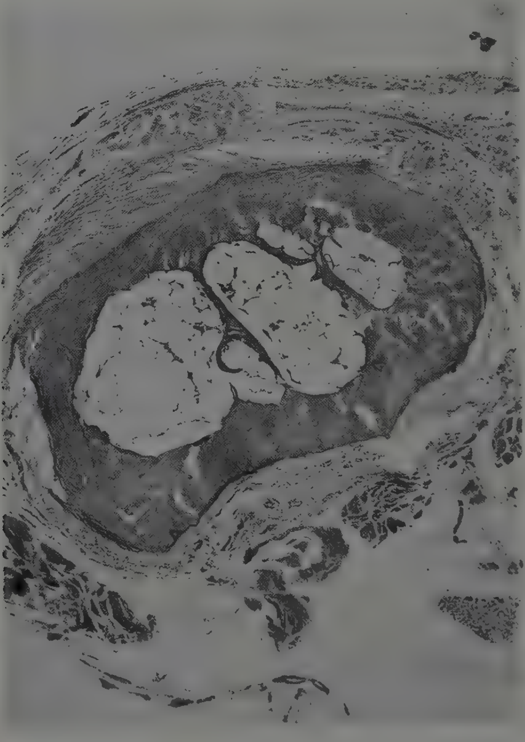

Figure 4.—Metastatic ossification in subcutaneous area. Osteoma cutis-like. Full form bone formation is present with marrow elements × 75.

rat which had big areas of loss of hair. In the animals infected with *S. brevicaulis*, 1 case was observed which had a big nodule similar to that seen in the animals infected with *C. albicans* (fig. 1). Histological examination showed that the nodules were composed of calcium deposits. There was no evident inflammatory reaction, but the calcium deposits were surrounded by fibrous tissue (fig. 2). To evaluate if more extensive calcium deposits might be present x-ray pictures were taken. It may be seen that in 1 case calcium deposits were not limited to the skin, but were found in the organs along the digestive tract (fig. 3). On 1 occasion subcutaneous bone formation was observed. All components of the bone could be seen including marrow components (fig. 4).

## Discussion

From the time of the first case of Teissier in 1877 a number of cases of calcinosis have been reported. The rarity of the condition made comprehensive studies difficult, since most of the reports were limited to single cases. However, there are several reviews in the literature (Steinitz, 1931; Durham, 1927; Brooks, 1939). The first investigators who stressed that coexistence of calcinosis and scleroderma is not coincidental were Thibierge & Weissenbach (1911). Since then the coexistence of calcinosis and scleroderma has been frequently cited as Thibierge-Weissenbach syndrome. Rothstein & Welt (1936) in their study of calcinosis in infancy and childhood found that various infections frequently preceeded the condition. That trauma may play a role in the development of calcinosis was observed by several authors (Ducase, 1922; Memmesheimer, 1922; Stradiotti, 1910; Ratte, Faugeras & Gondy, 1959; Touzard & Villeneuve, 1955). Constant & Phillips (1954) induced diffuse calcifications of the heart and muscles by feeding cotton rats with partially purified diet deficient in magnesium. Selye (1957) produced cutaneous calcinosis with sclerosis in rats, a condition similar to clinical scleroderma, by daily injections of dihydrotachysterol in corn oil. The slight traumatiziation produced by plucking the hair in a circular patch increased the frequency of the appearance of the condition.

It is of interest that during the prolonged presence of *C. albicans* and *S brevicaulis* in the spleen of Wistar rats, calcinosis was observed. It seems to be the first case of experimental calcinosis so closely resembling a similar condition occurring in human beings. It may be that the fungi are triggering some mechanism of events. For example, it is known that polysaccharides of *C. albicans* have growth-stimulating capacities for *Mycobacterium tuberculosis* (Mankiewicz, Stackiewicz, & Litwak, 1958). However, it is also possible that observed changes were in direct relation to the introduced organisms since there were extensive calcified areas in the sites at which the organisms were injected into the spleen. The occurrence of the subcutaneous calcifications could be explained by the toxic action of the living or destroyed organisms.

## Résumé

La présence prolongée de *Candida albicans* et *Scopulariopsis brevicaulis* dans la rate de rats Wistar a permis d'observer des dépôts sous cutanés de sels de calcium. Les caractéristiques sont analogues à celles de la calcinose connue en pathologie humaine. Ces lésions se sont présentées sous forme circonscrite et diffuse. Un cas d'ossification métastatique a été également observé.

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## Arturo Nannizzi

A commemorative service for Arturo Nannizzi was held on 12th November 1961 in the Aula Magna of the University of Siena. The address was given by Professor Valerio Giacomini, Professor of Botany, University of Naples and the Committee of Honour was as follows:—Archbishop of Siena, Mario Ismaele Castellano; Prefect of the Province of Siena, Carmelo Oneto di S. Lorenzo; Rector of the University of Siena, Giuseppe Bianchini; President of the Provincial Administration of Siena, Virgilio Lazzeroni; Mayor of Siena, Ugo Bartalini; President of the Monte dei Paschi, Danilo Verzili; Founder-President of the Chigiana Academy of Music, Guido Chigi Saracini; Dean of the Faculty of Law, Alessandro Raselli; Dean of the Faculty of Medicine, Alberto Bencini; Dean of the Faculty of Pharmacy, Lidia Monti; Registrar of the University of Siena, Camillo Amic.

The life and work of Professor Nannizzi who was a mycologist of international repute and a most distinguished member of the University of Siena was illustrated by an exhibit.

Invitations to attend the service were extended to a number of the members of the International Society for Human and Animal Mycology.

J. C. Gentles

## Meeting of the Japanese Society for Medical Mycology

The Fifth Annual Convention of the Japanese Society for Medical Mycology was held in Sendai in the North East of Japan on 6th and 7th October 1961. Professor Y. Takahashi, Tohoku University, who was President introduced the guest speaker Dr. C. W. Emmons of the National Institutes of Health, U.S.A. Dr. Emmons presented a comprehensive paper on cryptococcosis and this was followed by other papers on the same subject. Dr. E. Tsubura, Osaka University dealt with mycological aspects, Dr. Y. Mikamo, Sanraku Hospital and Professor K. Fukushima, Yokohama Medical College with the clinical aspects and Professor T. Hara, Osaka Medical College with therapy. A symposium on antifungal compounds was also held and in this the mode of action of various compounds was dealt with by Dr. K. Iwata, Tokyo University, dermatological aspects by Professor K. Higuchi, Kyuchu University and the difficulties in the clinical field by Dr. H. Yamato, Okayama Saiseikai Hospital.

More than 60 other papers dealing with either mycological or clinical aspects of mycoses were presented and actively discussed. It was decided that the 6th Annual Convention would be held in Tokyo on 18th and 19th October 1962.

T. Tsuchiya

## SOCIETY NEWS

Members are reminded that subscriptions for 1962 are now due and should be paid without delay to the General Secretary, Dr. R. Vanbreuseghem, Institut de Médecine Tropicale, 155 rue Nationale, Antwerp, Belgium or sent direct to the Society Account No. 133.700, Banque d'Anvers, Meir 48, Antwerp. In accordance with the By-laws, Article I(1) the subscription for 1962 is 6.00 U.S. dollars (or equivalent in other currencies) and this includes receipt of *Sabouraudia* (Statutes ISHAM Article 5(2) ). It should be noted however that from the beginning of volume 2 *Sabouraudia* will be sent only to those members who have paid their subscriptions for the current year.

*Montreal Meeting of ISHAM.* As has been previously announced a meeting of the society will be held in Montreal in August, by kind permission of the Organizing Committee of the 8th International Congress for Microbiology, and it is hoped that as many members as possible will attend. The first paper-reading session will be held on Friday 17th August (*i.e.* before the opening of the main congress) and the general business meeting of the society on Sunday 19th August. Members are urged to consult the society Statutes and By-laws published in *Sabouraudia* volume 1, part 2, pages 124-128 if they wish to make nominations for the Executive Committee (Statutes, Article 8), raise any matters of business (Statutes, Articles 10(3), 15), or to present a paper (By-laws, Article 4(2) ).

In order to attend the Montreal meeting registration as a delegate to the 8th International Congress of Microbiology is necessary. Registration forms and forms of application for accommodation may be had on application to the Secretariat, 8th International Congress for Microbiology, 3574 University Street, Montreal, Canada. Since block reservations in hotels for congress delegates have been made only from 18th August it is particularly important for members of ISHAM to apply for reservations at an early date. University Residences will not be available until Saturday 18th August.

## INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

### INCOME AND EXPENDITURE ACCOUNT FOR THE YEAR ENDING 31ST DECEMBER 1961

| RECEIPTS | | |
|---|---|---|
| Balance in hand at 1st January 1961 | | |
| in Bank of Antwerp . . . . | 37,424 | |
| in Midland Bank of England (£2,000) | 293,476 | |
| | | 330,900 |
| Interest Bank of Antwerp . . . . . . | | 277 |
| Subscriptions | | |
| Members and Sustaining Member Labaz . . . . . . | 54,025 | |
| Sustaining Members | | |
| Glaxo Laboratories Ltd. 2 years (£100) . . . . . . | 14,000 | |
| Schering Corporation (£53 8 0) | 7,476 | |
| | | 75,501 |
| Belgian Francs | | 406,678† |

| PAYMENTS | | |
|---|---|---|
| Secretary's expenses | | |
| Printing of notepaper . . . . | 1,302 | |
| Postage etc. . . . . . . | 21,344 | |
| | | 22,646 |
| Cheques (not presented) . . . . . . | | 300 |
| Bank error . . . . . . . . . . | | 300 |
| Bank charges . . . . . . . . . . | | 278 |
| Subscription to Federation of International Association—Belgium . . . . . . . . . . | | 100 |
| To Editor for expenses (£35 2 0) . . . . . . | | 4,914 |
| * Loss on sum of £2,000, transferred to Midland Bank of England, resulting from change in values of currencies (plus bank charges) . . | | 13,476 |
| Balance at 31st December 1961 | | |
| in England (£2,118 6 9) | 296,568 | |
| in Belgium (£486 9 0) | 68,102 | |
| (£2,604 15 9) | | 364,670 |
| Belgian Francs | | 406,684† |

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* At the outset of political unrest in the Belgian Congo the General Secretary, with the approval of the Executive Committee, transferred £2,000 from Belgian account to the Midland Bank of England as a precautionary measure. At the time of transfer the rate of exchange was 146.5 B.fr. to £1 sterling. The present rate of exchange is 140 B.fr. to £1.

† The slight discrepancy in figures results from unavoidable errors in calculations from one currency to another.

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Contributors are requested to submit papers in English but articles written in French, German, Portuguese or Spanish will be considered without prejudice.

Papers for publication should be sent to the Editor or to a member of the Editorial Board. Submission of a paper will be understood to imply that it reports unpublished work and is not under consideration for publication elsewhere and that if accepted for publication by the journal will not be published again in the same form without the consent of the society.

Papers should be headed with the title of the paper, the names of the authors (male authors initials and surname, female authors one forename in full) and the name and address of the institution where the work was carried out.

Two copies (top copy and one carbon) should be submitted in double-spaced typing and with 1½″ left-hand margin on quarto paper. A short comprehensive summary in English and also in one other language (French, German, Spanish, Portuguese) should be given on separate sheets. The position of tables and figures (submitted on separate sheets) should be indicated in the text. In the event of a MS. proving unacceptable the top copy together with any illustration will be returned.

Proposals for names of new taxa of fungi and actinomycetes must conform with requirements of the *International Code of Botanical Nomenclature* and the *International Code of Bacteriological Nomenclature* respectively. Every effort should be made to ensure that the scientific names used for fungi and bacteria are in line with the appropriate code.

Binomial names should be underlined in the text, the generic name must be given in full when first used in summary or text but thereafter may be abbreviated.

References in the text should be cited Brown & Smith (1956), or (Brown & Smith, 1956). Where a cited article has more than two authors the names of ali authors should be given when reference is first made in the text, *e.g.* (Brown, Smith & Jones, 1957) and subsequently as (Brown *et al.*, 1957). References at the end of the paper should be given in alphabetical order of the authors and should include the titles of the papers. Titles of journals should when possible be abbreviated in accordance with the *World List of Scientific Periodicals*, 3rd Ed. (1952).

Illustrations and diagrams should be large enough to allow for reduction. Diagrams should be drawn in indian ink on Bristol board complete with lettering numbers etc. If it is not possible to have the lettering and numbers included in the diagram, these should be printed on a transparent overlay attached to the diagram.

Drawings and photographs should include a statement of magnification before reduction. Photographs should be well contrasted prints on glossy paper. All tables, diagrams or photographs should have short legends describing their content (tables on the same sheet, diagrams and photographs on separate sheets). The legends should be comprehensible without reference to the text.

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